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**STUDIES ON THE REFOLDING
PROCESS OF OVALBUMIN**

MAKI ONDA

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ABBREVIATIONS

CD	circular dichloism
Tris	tris(hysroxymethyl)-aminomethane
EDTA	ethylenediamine tetraacetate
DTT	dithiothreitol
IAEDANS	N-iodoacetyl-N'-(5-sulfo-1-naphthyl)ethylenediamine
HPLC	high performance liquid chromqtography
SH condition	disulfide-reduced condition
SS condition	disulfide-bonded condition
IAA	iodoacetic acid
ANS	anilino-1-naphthalene-8-sulfonate

INTRODUCTION

Protein structure is constructed to fold a polypeptide chan spontaneously. Folding does not, however, occur by sampling all possible conformations randomly until the one with the lowest free energy is encountered (1). There are pathways during protein folding. To analyze the mechanism and pathway of folding is very important to understand the principle of protein structure construction.

A disulfide protein is a useful model for the investigation of protein folding mechanisms (2). Disulfide proteins that assume fully unfolded (3–12) or partially unfolded conformation (13–15) in their disulfide-reduced states can be oxidatively refolded with the aid of a chemical oxidant. The kinetic folding pathways of some small single-domain proteins, such as bovine pancreatic trypsin inhibitor (3–7), ribonuclease A (8), ribonuclease T1 (9), α -lactalbumin (10), and hirudin (11, 12) have been elucidated by identifying the disulfide structures in partially disulfide-bonded intermediates. A second class of disulfide proteins that assume a native-like conformation in the disulfide-reduced state has also been the target of protein folding studies. The influence of protein conformation on disulfide bond formation in the constant fragment of immunoglobulin light chain (16) and conversely, the influence of disulfide bonds in the folding kinetics of growth hormone (17), have been demonstrated.

Ovalbumin has unique structural characteristics as an alternative model for the second class of disulfide proteins. This egg white protein contains four cysteine sulfhydryls along with an intrachain disulfide in a single

polypeptide chain (18, 19). In urea-denatured state near neutral pH, intrachain sulfhydryl/disulfide exchanges occur and produce fifteen disulfide isomers theoretically (20). Furthermore, the egg white protein can refold to native state without recover of the native disulfide under the disulfide-reduced condition (21). These characteristics enable to analyze the refolding pathways and intermediates with tracing disulfide isomers without using oxidizing agents. A major problem encountered in the kinetic analysis of an oxidative refolding pathway is related to protein sulfhydryl accessibility to an oxidizing agent in the first step.

In this study, the author attempted to elucidate the refolding process of ovalbumin with analysis for intrachain disulfide rearrangements and disulfide isomers. In chapter I, two types of intermediates are detected during refolding process, and the author demonstrates the refolding pathway by means of kinetic analysis. In chapter II, it is declared that urea-denatured ovalbumin comprising non-native disulfide isomers correctly refold to native protein via sulfhydryl/disulfide exchange reactions. In chapter III, refolding intermediate is characterized by use of chemical modified ovalbumin with a non-native disulfide Cys 367- Cys 382, furthermore, the author discusses about refolding process and intra-molecular interaction govern the protein structure construction.

CHAPTER I

Refolding Process of Disulfide-bonded and reduced Ovalbumin from Urea-denatured States

Ovalbumin contains four cysteine sulfhydryls (Cys 11, Cys 30, Cys 367 and Cys 382) along with an intrachain disulfide (Cys 73-Cys 120) in a single polypeptide chain of 385 amino acid residues (18, 19). It has been shown that the conformational state of the disulfide-reduced ovalbumin is almost indistinguishable from that of the disulfide-bonded form (22). Furthermore, the egg white protein can refold from urea-denatured state in the SH condition, indicating spontaneous protein folding without the native disulfide bond (21). When disulfide-bonded ovalbumin denatured in a high concentration of urea is placed in non-denaturing conditions, the protein refolds much less efficiently than the disulfide-reduced form does (21). This may be related to the observation that many non-native disulfide isomers that have one disulfide and four sulfhydryls in a molecule are produced in a high concentration of urea (20). The detailed refolding mechanism, however, remains to be investigated for both disulfide-bonded and disulfide-reduced ovalbumin.

In the this chapter, the author investigated the refolding mechanism of ovalbumin in SS and SH conditions using the denatured protein state, D_A , as the starting protein sample. D_A was produced by incubation of the disulfide-bonded and disulfide-reduced ovalbumin in 9 M urea at pH 2.2, where possible sulfhydryl/disulfide exchange reactions in the disulfide-bonded protein are almost completely blocked (4). Refolding was

initiated by a pH jump procedure in which the denatured protein D_A is placed in a refolding buffer with near neutral pH. The author reports here that a partially folded intermediate state, I_N , is formed very rapidly in either the SS or SH condition as detected by far UV CD and intrinsic tryptophan fluorescence spectra. After the initial burst phase, the time course for the conformational regain followed biphasic kinetics in the SS condition, but it followed monophasic kinetics in the SH condition. According to the data from a peptide mapping analysis, the native disulfide in D_A undergoes nonspecific disulfide rearrangements at an early refolding stage in the SS condition and then is recovered during the subsequent slow refolding. These data along with other findings are consistent with a refolding mechanism for ovalbumin which includes nonproductive side chain-side chain interactions in the early intermediate I_N , which requires structural reorganization for subsequent correct refolding.

EXPERIMENTAL PROCEDURES

Materials

Ovalbumin (A_1 -ovalbumin, diphosphorylated form) was purified from fresh egg white by crystallization in an ammonium sulfate solution and subsequent ion exchange column chromatography as described (23, 24). Diphenylcarbamyl chloride-treated trypsin (type XI) and chymotrypsin (type II) were purchased from Sigma. *Achromobacter* proteinase I (EC 3.4.21.50) was obtained from Wako Pure Chemical Industries.

Denaturation and Refolding of Ovalbumin

For the refolding in the SS condition, denatured ovalbumin D_A was prepared by incubating the native, disulfide-intact protein at 1.0 mg/ml, at 37°C for 30 min in 0.25 M HCl containing 1.0 mM Na-EDTA and 9 M urea, pH 2.2. Refolding was initiated at 25 °C by 20-fold dilution of D_A with buffer A (50 mM Tris-HCl buffer, pH 8.6, 1.0 mM Na-EDTA) giving a final pH value of 8.2. The proteins were allowed to refold at 25 °C and were then analyzed by intrinsic tryptophan fluorescence and CD spectrum. The buffers were degassed at reduced pressure and equilibrated under N_2 atmosphere prior to the refolding. An equilibrium intermediate I_A was produced by 20-fold dilution of D_A with 50 mM potassium phosphate buffer, pH 2.2, containing 1.0 mM Na-EDTA. The equilibrium intermediate was placed for refolding in a near neutral pH condition, pH 8.2, by being mixed with 0.02 volume of 3.0 M Trizma (Tris) base.

For the experiments in the SH condition, the native disulfide in ovalbumin was fully reduced at 10 mg/ml by incubation with 15 mM dithiothreitol at 37 °C for 2 h in 50 mM Tris-HCl buffer, pH 8.2, containing 1.0 mM Na-EDTA (22). Disulfide-reduced D_A was produced by 10-fold dilution of the native, disulfide-reduced protein with 0.25 M HCl containing 1.0 mM Na-EDTA and 9 M urea, pH 2.2. The refolding from D_A , the preparation of I_A , and the refolding from I_A in the SH condition were carried out in the same ways as in the SS condition, except that all of the diluents contained 0.25 mM dithiothreitol.

Measurement of Intrinsic Tryptophan Fluorescence

The fluorescence spectrum of ovalbumin was measured with a fluorescence spectrophotometer (Hitachi, model F-3000). The intrinsic tryptophan residues in ovalbumin were excited at 295 nm, and emission spectrum was recorded at a wavelength range from 300 to 420 nm. All measurements were carried out at a constant temperature of 25 °C. The time course of fluorescence intensity change was monitored at 338 nm emission. For the spectrum measurements at an early refolding time, the time course of fluorescence intensity changes was monitored at various emission wavelengths with excitation at 295 nm, and data at a refolding time of 10 s were plotted.

CD Spectrum Measurement

The far UV CD spectrum was recorded at 25 °C with a spectropolarimeter (JASCO, J-720). The CD data were expressed as mean residue ellipticity (degrees·cm²/dmol) by using 111 as the mean residue weight of ovalbumin. CD spectra at a short refolding time were determined by measuring the time-dependent increase in CD ellipticities at various wavelengths, and the values at the refolding time 10 s were plotted as a function of wavelength. For rapid mixing experiments, D_A was applied to a stopped-flow rapid kinetics accessory (Applied Photophysics, RX.1000) attached to the same spectropolarimeter, and changes in CD ellipticity at 230 nm were recorded at 25 °C after an 11-fold dilution with buffer A. The mixing dead time that was determined by using 2,6-

dichlorophenolindophenol and L-ascorbate (25) was 20 ms.

Differential Scanning Calorimetry

D_A was refolded for 20 h under the SS and SH conditions, concentrated about 15-fold using a concentrator (Amicon, Centriprep-10), and passed through a Sephadex column (NAP-10, Pharmacia Biotech Inc.) equilibrated with 10 mM sodium phosphate buffer, pH 6.0. Overall recoveries from the original D_A forms were about 70%. The refolded proteins and corresponding native protein controls were analyzed with a differential scanning calorimeter (Micro Cal, MCS-DSC). The protein concentration was 0.4 mg/ml in 10 mM sodium phosphate buffer, pH 6.0. The temperature was scanned at 1 K·min⁻¹.

Analyses for Disulfide-involved Half-cystines

At various refolding times in the SS condition, sulfhydryl/disulfide exchanges were quenched by mixing the protein samples with 0.24 volume of 2 M HCl. Disulfide-involved half-cystines were determined by alkylation with a fluorescent reagent *N*-iodoacetyl-*N*9-(5-sulfo-1-naphthyl)ethylenediamine ; IAEDANS, and a subsequent peptide mapping procedure as described (20). Briefly, the acid-quenched protein was neutralized and alkylated with 0.1 M iodoacetamide in 9 M urea solution. The alkylated protein was precipitated in a cold acetone-HCl solution, dissolved in a urea solution, reduced with dithiothreitol, and then modified with IAEDANS. The modified protein was extensively proteolyzed with

the combination of trypsin, chymotrypsin, and *Achromobacter* protease I. The resultant peptides were analyzed by reversed phase HPLC with fluorescence monitoring (excitation, 340 nm; emission, 520 nm). For the standard experiment, the intact ovalbumin was fully reduced with dithiothreitol, all cysteine residues were labeled with IAEDANS, and the molecule was proteolyzed in the same way.

RESULTS

Folding Intermediate Detected by Optical Methods

Ovalbumin contains three tryptophan residues, Trp 148 in helix F, Trp 184 as the nearest neighbor residue of the COOH terminus of strand 3A, and Trp 267 in helix H (26). The conformational states of different forms of ovalbumin were analyzed by the intrinsic tryptophan fluorescence spectrum. As shown in Fig. 1, the fluorescence emission spectrum of native ovalbumin was indistinguishable for the disulfide-reduced and disulfide-bonded forms; the native proteins showed an emission maximum at 338 nm. D_A showed a typical red shift spectrum of an unfolded protein; the emission maximum was shifted to a longer wavelength of 352 nm, and the fluorescence intensity was decreased to 32% of the native form. When D_A was refolded at a near neutral pH, the protein showed at the early refolding time of 10 s a fluorescence spectrum that had a peak at the same wavelength but with much decreased intensity (57%) compared with the spectrum of native ovalbumin.

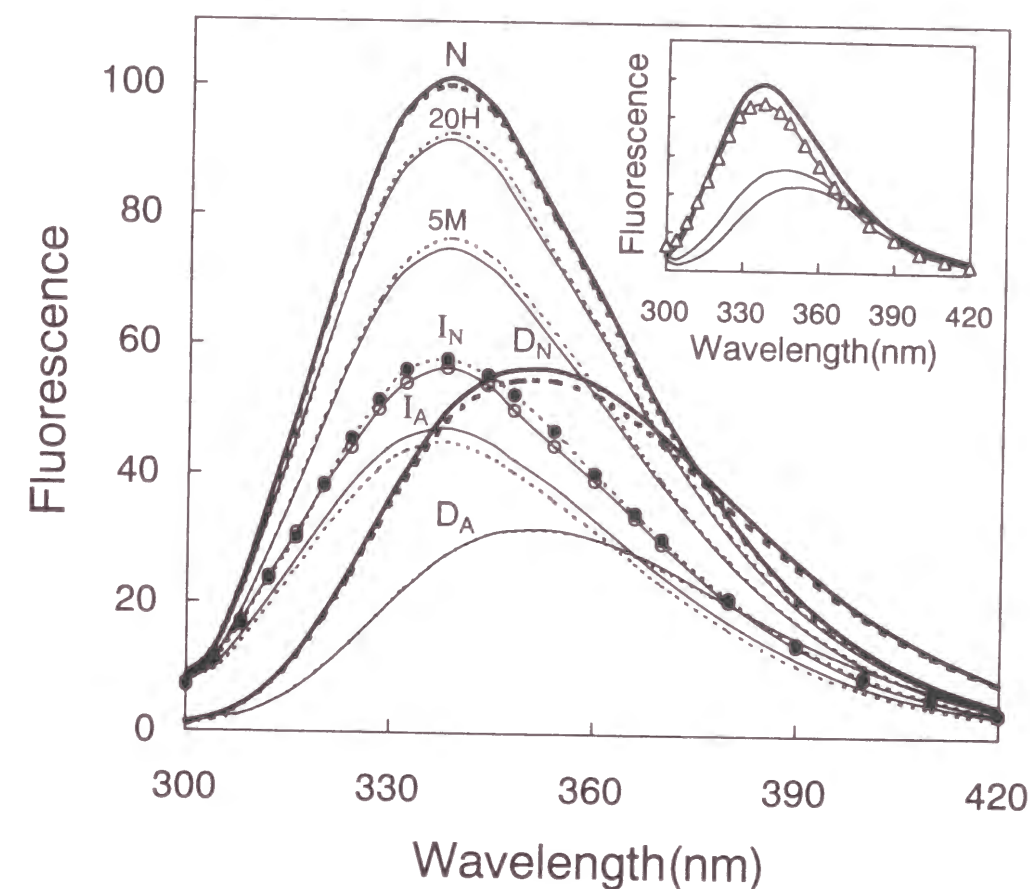


FIG.1. Fluorescence emission spectra of various states of ovalbumin.

In the SS (*solid lines*) and SH (*dotted lines*) conditions, the tryptophan residues in various states of ovalbumin were excited at 295 nm, and the emission spectra were recorded at 25 °C as described under “Experimental Procedures.” N (*thick lines*) represents native ovalbumin. D_A (*thin lines*) and D_N (*thick lines*) are the proteins denatured by protein incubation in 9 M urea at 37 °C for 30 min at pH 2.2 and at pH 8.2, respectively. The equilibrium intermediate state I_A (*thin lines*) was produced by 20-fold dilution of D_A with a non-denaturing buffer, pH 2.2. The intrinsic tryptophan fluorescence of the proteins refolded from D_A for 5 min (5 M, *thin lines*) and for 20 h (20H, *thin lines*) was recorded in the same way. For the fluorescence spectra at an early refolding time (I_N), the time course of fluorescence changes was recorded during refolding in the SS (*open circles with thin solid line*) and SH (*closed circles with thin dotted line*) conditions at different emission wavelengths, and the values at the refolding time of 10 s were plotted as a function of emission wavelength. In the *inset* the fluorescence spectra at pH 8.2 of the native protein (*thick solid line*) and the protein denatured in 9 M urea for 10 s (*open triangles with a thin line*) and for 5 and 30 min (*thin lines from top to bottom*) were recorded in the same way. The fluorescence intensity is shown by an arbitrary unit.

The fluorescence intensity was then increased at 5 min of the refolding and reached more than 90% of the value for the native protein at a prolonged refolding time of 20 h. The blue shifted spectrum observed at the early refolding time could be accounted for not by a solvent effect of decreased urea concentration, but rather by a change in protein conformation, since the maximum wavelength of fluorescence was the same 338 nm at the early stage (10 s) of the urea-induced denaturation of ovalbumin (Fig. 1, *inset*).

The same approach was carried out using far UV CD spectrum as a conformational probe. As shown in Fig. 2, native ovalbumin again showed almost the same spectrum in the SS and SH conditions. At the early stage (10 s) of the refolding in the SS and SH conditions, ovalbumin showed a CD spectrum that had 60% absolute ellipticity at 222 nm of the native form. The absolute ellipticity increased further with time of refolding, and the CD spectra of the proteins refolded for a prolonged time of 20 h were almost exactly the same as those of the native proteins. When the time course of CD ellipticity changes in the SS condition was monitored using a rapid mixing tool, the obtained data, while noisy, were consistent with that I_N formation from D_A is finished within the mixing dead time of 20 ms (Fig. 2, *inset*). The same was also true for the I_N formation in the SH condition (data not shown).

These data indicated that the partly folded intermediate I_N is formed very rapidly as an initial burst during the refolding in the SS and SH conditions at the near neutral pH; the intermediate had structural characteristics of 60% absolute CD ellipticity at 222 nm and 57% fluorescence intensity at

338 nm of the native form. Figs. 1 and 2 also demonstrate that D_A was transformed by 20-fold dilution with potassium phosphate buffer, pH 2.2, into a partially folded form I_A .

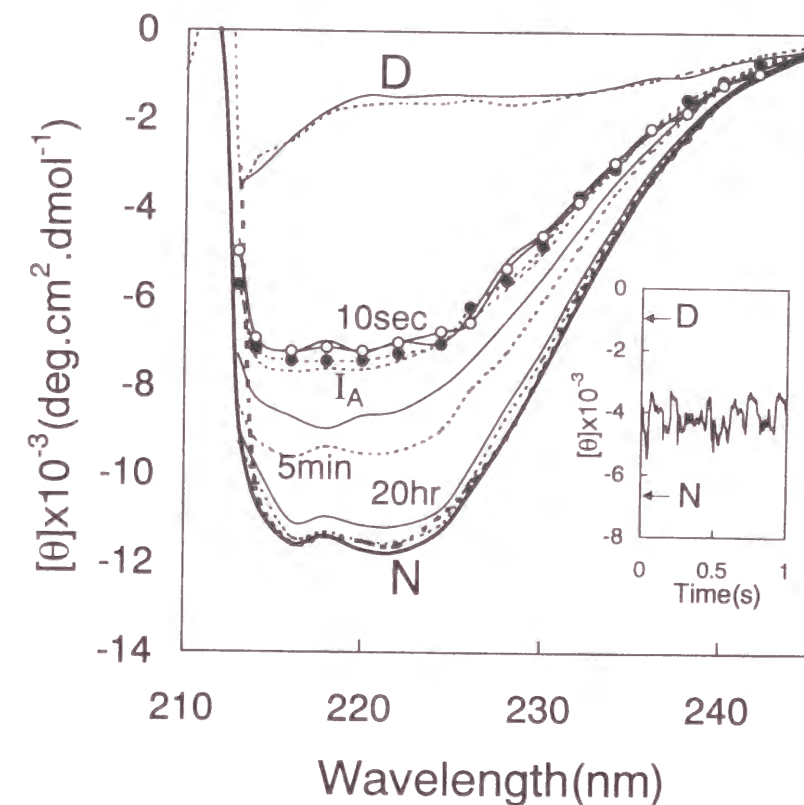


FIG.2. Far UV CD spectra.

In the SS (*solid lines*) and SH (*dotted lines*) conditions, the far UV CD spectra in various states of ovalbumin were recorded at 25 °C as described under “Experimental Procedures.” The designations of N, D_A , I_N , I_A , 5M, and 20H for the different ovalbumin states are the same as in Fig. 1. For the CD spectra for early refolding intermediates, the time course of changes in CD ellipticities at different wavelengths were recorded during refolding in the SS (*open circles with thin solid line*) and SH condition (*closed circles with thin dotted line*), and the values at the refolding time of 10 s were plotted as a function of wavelength. In the *inset*, the urea-denatured disulfide-bonded D_A was refolded at pH 8.2, and the changes in CD ellipticities at 230 nm were recorded for 1 s using a stopped-flow tool as described under “Experimental Procedures”; 230 nm was employed because of less noisy CD ellipticities at this wavelength than at a peak wavelength of 222 nm. D and N shown by *arrows* represent the CD ellipticities at 230 nm of D_A and of the native protein, respectively.

The far UV CD spectrum of I_A was almost indistinguishable from that of the early folding intermediate I_N . The fluorescence spectrum of I_A showed the maximum at the same wavelength (338 nm) as that of I_N . The decreased fluorescence intensity for I_A can be accounted for by solvent effects by acid, since D_A shows decreased fluorescence intensity compared with the urea-denatured ovalbumin at pH 8.2 (Fig. 1). In addition, a previous report has shown that native ovalbumin represents significantly decreased fluorescence (about 55%) at pH 2.2 from that at pH 8.2 but almost indistinguishable far UV CD spectra at the two different pH values (27). The disulfide rearrangements should be almost completely blocked at pH 2.2 (4). Furthermore, the conformational state was constant during 2-h incubation at pH 2.2, as evaluated by the far UV CD analysis (data not shown). The author therefore concluded that the acid-quenched I_A is an equilibrium intermediate state that possesses structural characteristics equivalent to I_N .

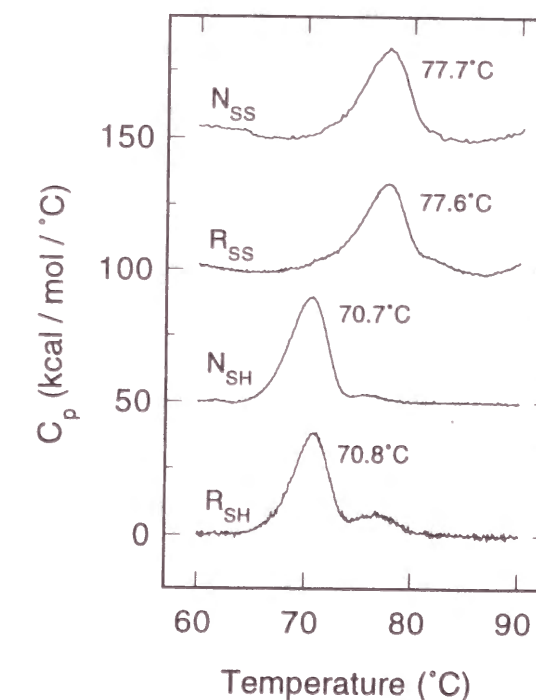
Differential Scanning Calorimetry Analysis of Refolded Ovalbumin

The data from the intrinsic fluorescence and far UV CD analyses showed that most if not all ovalbumin molecules refold into the native state at 20 h incubation (Figs. 1 and 2). The integrity of native refolding was investigated more rigorously by an alternative method of differential scanning calorimetry. Fig. 3 demonstrates that the protein refolded for 20 h in the SS condition underwent thermal transition with almost the same melting temperature as did the native protein counterpart; the thermal

denaturation temperatures were 77.7 °C for the native protein and 77.6 °C for the refolded protein. Likewise, the melting temperature for the protein refolded in the SH condition (70.8 °C) was almost the same as that for native, disulfide-reduced ovalbumin (70.7 °C). The minor peak observed for the refolded protein in the SH condition may be accounted for by reoxidation of the native disulfide during the refolding time of 20 h and/or during the protein concentration procedure prior to the calorimetry analyses, since the peak temperature was about 77 °C, which is essentially the same value as the melting temperature for the native, disulfide-bonded protein. The peak area was, however, less than 8% of the major peak. The author therefore concluded that most of the ovalbumin molecules can refold correctly in the SS and SH conditions into the same protein energy states as the native proteins.

FIG.3. Differential scanning calorimetry analysis of refolded ovalbumin.

Ovalbumin refolded from D_A for 20 h and native protein controls were analyzed by differential scanning calorimetry in 10 mM sodium phosphate buffer, pH 6.0, with a temperature scanning rate of 1 K · min⁻¹. Endothermic transition profiles for the native, disulfide-bonded protein (NSS), for the native, disulfide-reduced protein (NSH), for the protein refolded in the SS condition (RSS), and for the protein refolded in the SH condition (RSH) are arbitrarily shifted on the ordinate scale for clarity.



Refolding Kinetics after the Initial Burst Phase

The data in Figs. 1 and 2 also show that slow refolding proceeds after rapid formation of the intermediate state. The time courses of the refolding from D_A after the initial burst were examined by intrinsic fluorescence and CD analyses. Fig. 4 (*panels A and B*) shows the time-dependent increases in the fraction of the re-folded protein after 6 s of the refolding from D_A . The refolding in the SS condition apparently followed biphasic kinetics (for the quantitative calculation, see “Discussion”). In contrast, ovalbumin refolded with simple monophasic first-order kinetics in the SH condition. No significant difference was observed in either the SS or SH condition for the kinetic profiles obtained by the fluorescence and CD analyses.

The data in Figs. 1 and 2 demonstrate that D_A is transformed by dilution with potassium phosphate buffer, pH 2.2, into a partially folded form I_A , which has structural characteristics equivalent to those of I_N . To examine whether or not I_A can refold in a manner similar to the refolding from D_A , the acid-quenched equilibrium intermediate was placed in a near neutral condition, and the time course for the refolding was monitored by intrinsic fluorescence and CD analyses. As shown in the *panels C and D* of Fig. 4, I_A refolded with essentially the same kinetics as D_A ; the refolding followed biphasic kinetics in the SS condition but monophasic kinetics in the SH condition.

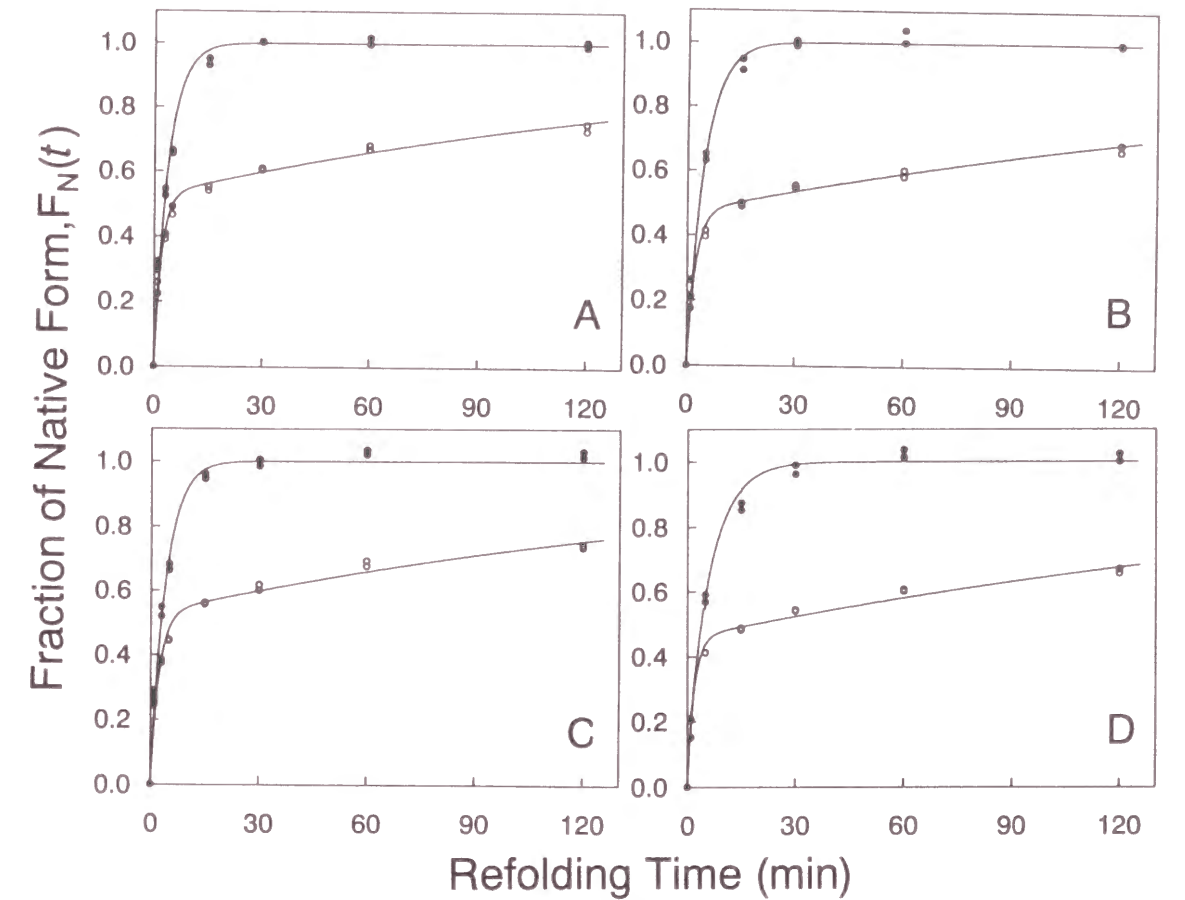


FIG.4. Time course for the refolding after the initial burst phase.

D_A and I_A were prepared as described under “Experimental Procedures”. The time course for the refolding at pH 8.2, 25 °C from D_A (*panels A and B*) and from I_A (*panels C and D*) in the SS (*open circles*) and SH (*closed circles*) conditions were monitored by the intrinsic tryptophan fluorescence at 338 nm (*panels A and C*) and by CD ellipticity at 222 nm (*panels B and D*) as described under “Experimental Procedures”. The *ordinate* shown by $F_N(t)$ represents the fraction of the native form at the refolding time t , which was calculated by the equation $F_N(t) = (X_0 - X_t)/(X_0 - X_N)$, where X_0 and X_t are the values at the refolding times of 6 s and t , respectively. X_N is the final value of the refolding; the fluorescence intensity and CD ellipticity at the refolding time of 20 h were employed as X_N . As control experiments, disulfide-bonded and disulfide-reduced ovalbumin preincubated at pH 2.2 in the absence of urea were diluted 20-fold with buffer A giving the same final pH value of 8.2; the CD and fluorescence intensities were confirmed to show invariably the native values during the incubation time of 120 min. The *solid curves* represent nonlinear least squares fits of the duplicate experimental data to theoretical rate equations (Equations 1 and 2, described under “Discussion”). For the nonlinear least squares fits, a program utilizing a Levenberg-Marquardt algorithm was employed.

Sulfhydryl/Disulfide Exchanges during Refolding

The data in Fig. 4 indicate that the biphasic kinetics are closely related to the presence of the intrachain disulfide bond. The denatured state D_A should contain the native disulfide Cys 73-Cys 120 and four cysteine residues Cys 11, Cys 30, Cys 367 and Cys 382 since intrachain sulfhydryl/disulfide exchange reactions possible for fully denatured ovalbumin (20) should be almost completely quenched at the employed condition of pH 2.2 (4). The question, however, arose as to whether the involvement of disulfide rearrangement reactions during the refolding at pH 8.2 results in the more complex kinetics in the SS condition than in the SH condition. To examine the possibility of disulfide rearrangements during the refolding, the author determined the disulfide-involved half-cystines by the peptide mapping procedure at various refolding times. As shown in Fig. 5A, only Cys 73 and Cys 120 were detected as disulfide-involved cysteines at the refolding time 0 from D_A . The disulfide-involved Cys 73 and Cys 120, however, decreased significantly at an early refolding time of 1.0 min; concomitantly at this stage, all of the other four cysteines, Cys 11, Cys 30, Cys 367 and Cys 382, were detected as the disulfide-involved cysteines. The amounts as disulfide-involved cysteines were the minima (about 60%) for Cys 73 and Cys 120 but the maxima (about 20%) for the other cysteines at 15 min of the refolding. The disulfide-involved Cys 73 and Cys 120, however, both increased gradually after 15 min, and the amounts were about 90% at 20 h refolding.

The next question was related to the refolding stage that involves the nonproductive disulfide rearrangements. In the refolding system from D_A , ovalbumin denatured under acid-urea conditions was placed in near neutral buffer conditions to be allowed to refold. The original D_A , therefore, may first be transiently transformed by pH jump to the highly denatured state at pH 8.2 and then to the early intermediate state I_N by conformational conversion. Such a highly denatured state should undergo extensive disulfide rearrangements at near neutral pH (20). However, the data in Figs. 1, 2 and 5A showing that the rapid formation of I_N precedes the disulfide rearrangement appeared to support the rearrangement reactions in I_N rather than in the transient denatured state. To confirm this, the author analyzed disulfide-involved cysteines during the refolding from I_A , which has the native disulfide and equivalent structural characteristics to I_N , since the involvement of ovalbumin in the transient denatured state can be skipped in this refolding system. The data in Fig. 5B demonstrate that the disulfide-involved Cys 73 and Cys 120 decreased at an early refolding time in almost the same manner as in the direct refolding from D_A . The disulfide-involved Cys 73 and Cys 120 showed the minima at 15 min and 5 min, respectively, and then both increased gradually; at 20 h of the refolding, the amounts again reached values of about 90%. Almost the same time courses as in the direct refolding from D_A were also observed for the other four cysteine residues; the disulfide-involved Cys 11, Cys 30, Cys 367 and Cys 382 increased tentatively at an early refolding time and then decreased with the time of the refolding.

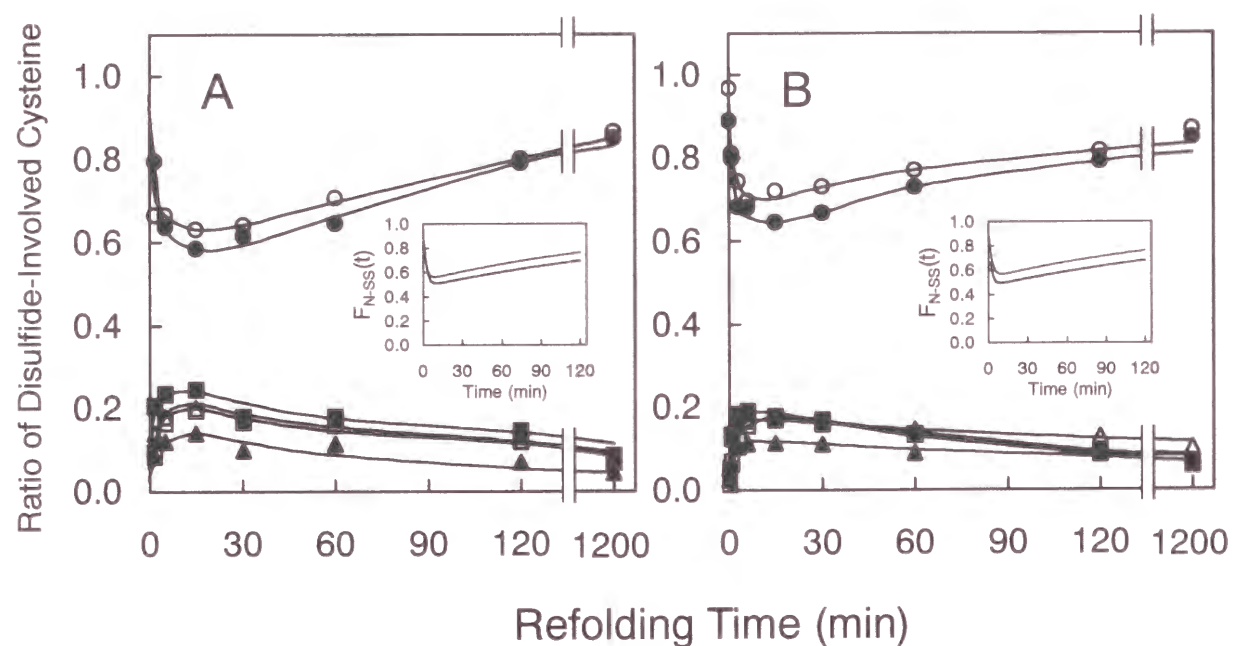
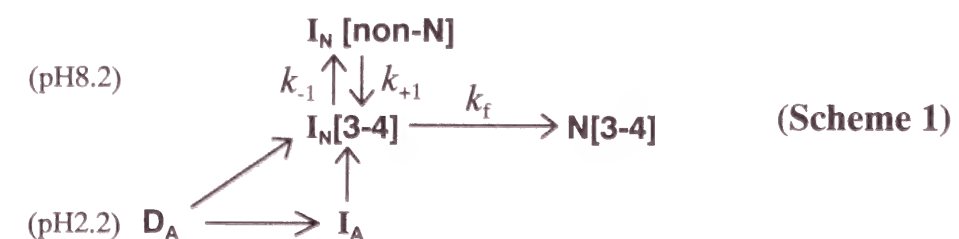


FIG.5. Disulfide rearrangements during the refolding.

In *panel A*, D_A prepared as described under “Experimental Procedures” was refolded at pH 8.2, 25 °C. In *panel B*, I_A that had been prepared by 20-fold dilution of D_A with potassium phosphate buffer, pH 2.2, was refolded at 25 °C after being mixed with a 0.02 volume of 3 M Tris base, giving a final pH value of 8.2. At various refolding times in the SS condition, the disulfide-involved cysteines for Cys 11 (*open squares*), Cys 30 (*closed squares*), Cys 73 (*closed circles*), Cys 120 (*open circles*), Cys 367 (*closed triangles*), and Cys 382 (*open triangles*) were determined using a peptide mapping analysis (20). The data are the averages for duplicate determinations. The *insets* present the theoretical time courses for $F_{N-SS}(t)$ which is defined as the fraction of the ovalbumin species with the native disulfide Cys 73-Cys 120; $F_{N-SS}(t)$ was calculated using Equation 3 and the refolding rate constants in Table 1 described under “Discussion.” The *solid* and *dotted curves* were obtained by calculation using the rate constants from the fluorescence and CD analyses, respectively. The theoretical curves for the refolding from D_A (*panel A*) show the minimum values at 9.0 and 10.5 min for the fluorescence and CD data, respectively. For the refolding from I_A (*panel B*), $F_{N-SS}(t)$ was the minimum at 10.3 min for the fluorescence data and at 9.2 min for the CD data.

DISCUSSION

The data in the present study demonstrate that most, if not all, of the ovalbumin molecules in D_A refold correctly in either the SS or SH condition into the native form, N, as evaluated by the intrinsic fluorescence and far UV CD spectra (Figs. 1 and 2). Furthermore, the results from the differential scanning calorimetry analysis provide evidence for the equivalent conformational stability of the refolded protein to the native protein counterpart (Fig.3). To my knowledge, this is the first demonstration that the integrity of native refolding was confirmed by differential scanning calorimetry for such a large protein as ovalbumin. An intermediate state I_N is produced from D_A at an early stage (within 20 ms) of the refolding at pH 8.2 (Figs. 1 and 2) in either the SS or SH condition. An equivalent equilibrium intermediate I_A is formed when D_A is diluted with a non-urea acidic buffer (Figs. 1 and 2). As unexpected findings, the native disulfide Cys 73-Cys 120 undergoes nonproductive disulfide rearrangements due to sulfhydryl/disulfide exchanges with the other four cysteines during the refoldings from D_A and I_A in the SS condition (Fig. 5). These data are consistent with the following Scheme for the refolding process of urea-denatured ovalbumin,



where $I_N[\text{non-N}]$ represents the mispaired-disulfide intermediate that is formed during the refolding in the SS condition. $I_N[3-4]$ is the intermediate with the native disulfide Cys 73-Cys 120 (SS condition) or without any intrachain disulfide (SH condition) and assumed to be the competent one for subsequent folding into N[3-4] with a first-order rate constant, k_f . Through the disulfide rearrangements, $I_N[\text{non-N}]$ undergoes reversible interconversion with $I_N[3-4]$; k_{+1} and k_{-1} are the first-order rate constants for the conversions from $I_N[\text{non-N}]$ to $I_N[3-4]$ and from $I_N[3-4]$ to $I_N[\text{non-N}]$, respectively. The *small arrows* with *thin solid lines* represent the initial burst for the conformational transition and/or pH jump steps.

Scheme 1 is consistent with the kinetic data of the refoldings after the initial burst phase (Fig. 4). $F_N(t)$, which is defined as the fraction of N[3-4] at a refolding time t , can be expressed in the SS condition as follows.

$$F_N(t) = 1 + \frac{k_f + r_2}{r_1 - r_2} \exp(r_1 t) - \frac{k_f + r_1}{r_1 - r_2} \exp(r_2 t) \quad (\text{Eq. 1})$$

where r_1 and r_2 are related to the rate constants as $r_1 = -\alpha + \sqrt{\alpha^2 - \beta}$, $r_2 = -\alpha - \sqrt{\alpha^2 - \beta}$, $2\alpha = k_{+1} + k_{-1} + k_f$, and $\beta = k_{+1} k_{-1}$. In the SH condition, no disulfide rearrangements are involved ($k_{+1} = k_{-1} = 0$). Equation 1 can therefore be reduced into a single exponential first-order rate equation.

$$F_N(t) = 1 - \exp(-k_f t) \quad (\text{Eq. 2})$$

As displayed by the *solid lines* in Fig. 4, the data for the refolding from either D_A or I_A can be fitted quite adequately to Equation 1 in the SS

condition and to Equation 2 in the SH condition. The first-order rate constants obtained by the data fitting analysis are summarized in Table 1.

TABLE 1
Rate constants implicated for the ovalbumin refolding

The first-order rate constants for Scheme 1 were obtained by fitting the refolding data in Fig. 4 to Equation 1 in the SS condition and to Equation 2 in the SH condition.

Refolding from	Conditions	Conformational probe ^a	Rate constants (min^{-1})		
			k_{+1}	k_{-1}	k_f
D_A	SS	FL	0.011	0.23	0.27
I_A	SS	FL	0.010	0.19	0.23
D_A	SH	FL			0.25
I_A	SH	FL			0.24
D_A	SS	CD	0.0094	0.23	0.21
I_A	SS	CD	0.0092	0.28	0.24
D_A	SH	CD			0.21
I_A	SH	CD			0.16

^a FL and CD represent the fluorescence and CD spectrum analysis, respectively

The three first-order rate constants each show, in either the SS or SH condition, almost equivalent values for the two refolding systems (from D_A and I_A) and for the two conformational probes, reinforcing the present refolding mechanism of Scheme 1. The obtained rate constants appear also reasonable for the elucidation of the time course for the disulfide rearrangements in Fig. 5. According to Scheme 1, $F_{N-SS}(t)$, which is defined as the ovalbumin fraction with the native disulfide at refolding time t , should correspond to the sum of the fractions of N and I_N . $F_{N-SS}(t)$ can therefore be expressed as follows.

$$F_{N-SS}(t) = 1 - \frac{k_{-1}}{r_1 - r_2} \exp(r_1 t) + \frac{k_{-1}}{r_1 - r_2} \exp(r_2 t) \quad (\text{Eq. 3})$$

The fraction of ovalbumin species with the native disulfide can therefore be calculated as a function of t using the rate constants in Table 1 as displayed in the *insets* of Fig. 5. The value for the disulfide-involved Cys 73 and Cys 120, which also includes their participation in mispaired disulfides with the other cysteines, should not be exactly the same as, but should be closely related to, the amount of the protein species with the native disulfide Cys 73-Cys 120. Fig. 5 represents that the theoretical curve is compatible with the experimental data at the qualitative level. The values for $F_{N-SS}(t)$ decrease rapidly at early refolding times showing the minima at 9–10 min and then gradually increasing with the time of the refolding. Such theoretical profiles were very similar to the experimental time courses for the disulfide-involved Cys 73 and Cys 120, which show the minima at 5–15 min.

The results from the data-fitting analyses provide important information about the refolding mechanism of ovalbumin. First, the refolding of ovalbumin follows biphasic kinetics in the SS condition (Fig. 4). As a general mechanism for biphasic refolding kinetics, the involvement of the parallel pathway that is related to *cis-trans* isomerization of proline residues has been demonstrated (28). Ovalbumin indeed contains 14 proline residues. Scheme 1 is, however, consistent with the biphasic kinetics in the SS condition without including parallel pathways. A decelerated folding mechanism in disulfide-bonded ribonuclease T1 which

is accounted for by decreased proline isomerization rate (29) is also unlikely in the refolding of ovalbumin, since the folding rate constant k_f is almost identical in the SS and SH conditions (Table 1). It is, therefore, very likely for the refolding kinetics of ovalbumin that the effects of the proline isomerization problem, if any, work in an undifferentiated manner in the SS and SH conditions. Second, according to the author's previous simulation analysis for the disulfide rearrangements in ovalbumin, the rate constant k for a sulfhydryl/disulfide exchange in 9 M urea at pH 8.2 is related to the equation $k = 820 n_e^{-2}$, where n_e is the "effective number" of amino acids separating a disulfide and a relevant sulfhydryl (20). For the initial disulfide rearrangements by the nucleophilic attacks of the four cysteines on the native disulfide Cys 73-Cys 120, the rate constants should be in the order Cys 30 > Cys 11 > Cys 367 > Cys 382; the absolute rates for the productions should be quite variable from the most rapid reaction (attack by Cys 30 forming Cys 30-Cys 73 and Cys 30-Cys 120) of 0.44 min⁻¹ to the slowest reaction (attack by Cys 382 forming Cys 73-Cys 382 and Cys 120-Cys 382) of 0.012 min⁻¹. As shown in Table 1, the rate constant for the formation of mispaired disulfides in I_N (k_{-1}) is approximately half of that for the most rapid reaction in the highly denatured state. Although the value for k_{-1} is an average value for many rearrangement reactions, the data in Fig. 5 represent almost equivalent disulfide participation for Cys 11, Cys 30 and Cys 382, except for a slightly lower value for Cys 367. These data may reflect some compact but highly flexible conformational state for I_N in which the effective concentrations of

the four cysteines sulfhydryls relative to the native disulfide are all significantly high.

The present refolding mechanism of ovalbumin has implications for the current view of the nature of protein folding intermediates. The equilibrium molten globule state has been observed with several globular proteins as a partially denatured form; the state has been claimed to be a general productive intermediate in protein folding (30–32). There are, however, examples in which protein folding proceeds via two-state behavior without an apparent folding intermediate (33, 34). Theoretical approaches for protein folding have suggested that folding intermediates accumulate because of kinetic traps caused by partial misfolding (35, 36). Indeed, experiments with cytochrome *c* represent that the refolding proceeds via an essentially two-state behavior without any apparent intermediate and that a trapped intermediate under three-state conditions results from misfolding of the polypeptide chain in the initial collapse step (37). In the ovalbumin refolding, I_N is formed as an initial burst, and the subsequent regain of the native conformation proceeds slowly (Figs. 1, 2 and 4). The observations that both the native disulfide species D_A and I_A undergo non-native disulfide rearrangements in an early stage of the refolding (Fig. 5) clearly demonstrate that nonspecific side chain-side chain interactions are involved in an early intermediate of ovalbumin refolding. The early intermediate I_N is therefore very likely to be a molecular collapse intermediate that requires subsequent structural reorganization for the correct refolding.

The data in Fig. 5 represent that the protein species with a mispaired disulfide can refold correctly into the native disulfide form via disulfide rearrangements. A sulfhydryl/disulfide protein that contains, in its natural or engineered form, both cystine and cysteine residues in the native molecule as in ovalbumin may generally work as a useful model for the investigation of a protein folding mechanism. The folding mechanisms of disulfide proteins have been investigated using mostly oxidative refolding systems in which the disulfide-reduced, denatured state is transformed into the native, disulfide-bonded form with the help of an oxidizing agent. A protein disulfide formation, however, includes multiple chemical steps: the first step is the intermolecular attack of a protein sulfhydryl on an oxidizing disulfide agent generating a protein mixed disulfide, and the second step is the intramolecular attack of a second protein sulfhydryl on the mixed disulfide (2). Intrachain sulfhydryl/disulfide exchanges also occur during the refolding of most of the small single-domain proteins (3–12). A major problem encountered in the kinetic analysis for an oxidative refolding pathway is related to the protein sulfhydryl accessibility in the first step to an oxidizing agent, as demonstrated for bovine pancreatic trypsin inhibitor (17). In the refolding process of the sulfhydryl/disulfide protein, however, only intrachain sulfhydryl/disulfide exchange reactions should be included. The sulfhydryl accessibility problem in the first reaction can therefore be circumvented.

CHAPTER II

Refolding of Urea-denatured Ovalbumin Comprises Non-Native Disulfide Isomers

In former chapter, the author demonstrated the refolding pathway of ovalbumin (see Scheme 1 in chapter I). It shows the native disulfide in the acid/urea-denatured ovalbumin undergoes nonspecific disulfide rearrangements in an initial burst intermediate and then is recovered during the subsequent slow refolding. Furthermore, the pathway shows that the non-native disulfide isomers possibly refold to native state via sulfhydryl/disulfide exchange reactions.

In this chapter, the author investigates whether or not the urea-denatured ovalbumin comprising non-native disulfide isomers, D[non-N], can refold into N[3-4] with the correct disulfide. As described in a previous report (20), fifteen non-native disulfide isomers are produced when disulfide-bonded ovalbumin is denatured at high concentrations of urea at near neutral pH. According to the X-ray crystallographic data (26), however, only the native disulfide Cys73-Cys120 can adopt the native conformation (Fig. 6). The refolding process of ovalbumin denatured in 9 M urea at pH 8.2 was investigated by using several different structural approaches. The author reports here that most, if not all, of the D[non-N] species can correctly refold into the native form via disulfide rearrangements according to an extended version of Scheme 1.



FIG. 6. Schematic view of ovalbumin. The figure is based on the X-ray crystallographic data of ovalbumin (26) and was drawn using the MolScript program (45). The numbered shaded spheres represent the sulfur atoms: 1, Cys11; 2, Cys30; 5, Cys367; 6, Cys382. The sulfur atoms 3 (Cys73) and 4 (Cys120) form the native disulfide bond.

EXPERIMENTAL PROCEDURES

Materials

A₁-ovalbumin was purified as described in former chapter. Diphenylcarbamyl chloride-treated trypsin (Type XI) and chymotrypsin (Type II) were purchased from Sigma. *Achromobacter* protease I (E.C. 3.4.21.50) was obtained from Wako Pure Chemical Industries.

Denaturation and Refolding of Ovalbumin

Denatured ovalbumin was prepared by incubating the native, disulfide-bonded protein at 1.0 mg/ml, 37°C for 30 min in buffer A (50 mM Tris-HCl buffer, pH 8.2, 1.0 mM Na-EDTA) containing 9 M urea. Refolding was initiated at 25°C by 20-fold dilution of the denatured protein with

buffer A, giving a final urea concentration as low as 0.45 M. The proteins were allowed to refold at the same temperature, and then analyzed for trypsin resistance, intrinsic tryptophan fluorescence and CD spectrum. An acid-quenched equilibrium intermediate was produced by 20-fold dilution of the urea-denatured protein with 50 mM K-phosphate, pH 2.2 containing 1.0 mM Na-EDTA. The buffers were degassed at reduced pressure and equilibrated under an N₂ atmosphere prior to the refolding experiments.

Measurement of Intrinsic Tryptophan Fluorescence and CD Spectrum

The fluorescence spectrum of ovalbumin was measured with a Hitachi fluorescence spectrophotometer (Model F-3000). The intrinsic tryptophan residues in ovalbumin were excited at 295 nm and the time course of fluorescence intensity change was monitored at 338 nm emission. The far-UV CD spectrum of ovalbumin was recorded with a spectropolarimeter (JASCO, J-720). All measurements were carried out at a constant temperature of 25°C. The manner of measurements and so forth were carried out as described in former chapter. For rapid mixing experiments, a stopped-flow rapid kinetics accessory (Applied Photophysics, RX.1000) attached to the same spectropolarimeter was employed and changes in the CD ellipticity of the urea-denatured ovalbumin at 230 nm were recorded at 25°C after 10-fold dilution with buffer A. The dead time for mixing, determined by using the reaction between 2,6-dichlorophenolindophenol and L-ascorbate (25), was 20 ms.

Trypsin-resistance Assay

Refolded ovalbumin with the native conformation is considered to be a trypsin resistant species as described (21). At various refolding times, the protein was mixed with 0.01 volume of different concentrations (1.25 or 12.5 mg/ml) of trypsin and digested at 25°C for 1 min. The digestion was terminated by addition of soybean trypsin inhibitor. The proteins were electrophoresed on a sodium dodecyl sulfate polyacrylamide gel (10% polyacrylamide/0.27% bisacrylamide) according to the standard method of Laemmli (41), and then stained with Coomassie brilliant blue R-250. The amount of trypsin-resistant ovalbumin was determined from the band intensity, which was measured with a densitometer (Shimadzu, CS-9000).

Analyses for Disulfide-involved Half-cystines

At various refolding times, sulfhydryl/disulfide exchanges were quenched by mixing the protein samples with 0.24 volume of 2 M HCl. Disulfide-involved half-cystines were determined by alkylation with a fluorescent reagent IAEDANS, and subsequent peptide mapping procedure as described elsewhere (20).

Determination of Cysteine Sulfhydryls by Amino Acid Analysis

The quenching of sulfhydryl/disulfide exchanges in the refolding protein by acid, alkylation of free cysteine sulfhydryls with iodoacetamide, and protein precipitation in a cold acetone-HCl solution were carried out in the same way as described in former. The alkylated protein was hydrolyzed

in 6 N HCl containing 0.1 % (v/v) phenol in the gas phase at 110°C for 20 h under vacuum. The carboxymethyl cysteine was determined with an amino acid analyzer (Hitachi, L-8500A).

Differential Scanning Calorimetry

The denatured protein was refolded for 20 h, concentrated about 15-fold using a concentrator (Amicon, Centriprep-10), and passed through a Sephadex column (Pharmacia Biotech, NAP-10) equilibrated with 10 mM Na-phosphate buffer, pH 6.0. Overall recoveries from the original denatured protein were about 70 %. The refolded protein and native protein control were analyzed with a differential scanning calorimeter (Micro Cal, MCS-DSC). The protein concentration was 0.4 mg/ml in 10 mM Na-phosphate buffer, pH 6.0. The rate of temperature change was 1 K·min⁻¹.

RESULTS

Folding Intermediate Detected by Far-UV CD and Intrinsic Tryptophan Fluorescence Spectra

In chapter I, the refolding mechanism of ovalbumin in either a disulfide-bonded or a disulfide-reduced condition was investigated using a starting denatured protein sample produced by protein incubation in 9 M urea at pH 2.2, where possible sulfhydryl/disulfide exchange reactions are almost completely blocked; the refolding was initiated by a pH-jump procedure in

which the denatured protein is placed in a refolding buffer of near-neutral pH. A partially folded intermediate state, I_N, has been shown to be formed in an initial burst phase, as detected by far-UV CD and intrinsic tryptophan fluorescence spectra, in either the disulfide-bonded or disulfide-reduced condition. The partially folded equilibrium intermediate with structural characteristics equivalent to those of I_N is formed upon the dilution of the acid/urea-denatured ovalbumin with an acidic buffer, pH 2.2.

In this chapter, the author examined similarly whether or not the initial burst intermediate is also formed during the refolding from the urea-denatured ovalbumin at near neutral pH, where different disulfide isomers should be present (20). As shown in Fig. 7, the refolding proteins from the urea-denatured state showed at the early stage of 10 s an intermediate CD spectrum that had 60% of the absolute ellipticity at 222 nm of the native form. When the time course of CD ellipticity change was monitored using a rapid mixing apparatus, the obtained data were consistent with completion of the formation of the intermediate from the urea-denatured state within the mixing dead time of 20 ms (Fig. 7, inset).

Ovalbumin contains three tryptophan residues, W148 in helix F, W184 as the nearest neighbor residue of the C-terminus of strand 3A and W267 in helix H (26). As shown in Fig. 8, the fluorescence emission spectrum of the native form had an emission maximum at 338 nm. The urea-denatured protein showed a typical red-shifted spectrum of an unfolded protein: the emission maximum was shifted to a longer wavelength of 352 nm, and the fluorescence intensity was decreased to 56 % of the native

form. When the urea-denatured ovalbumin was refolded, the protein showed at an early refolding time of 10 s a fluorescence spectrum that had a peak at the same wavelength, but with much decreased intensity as compared with the spectrum of native ovalbumin.

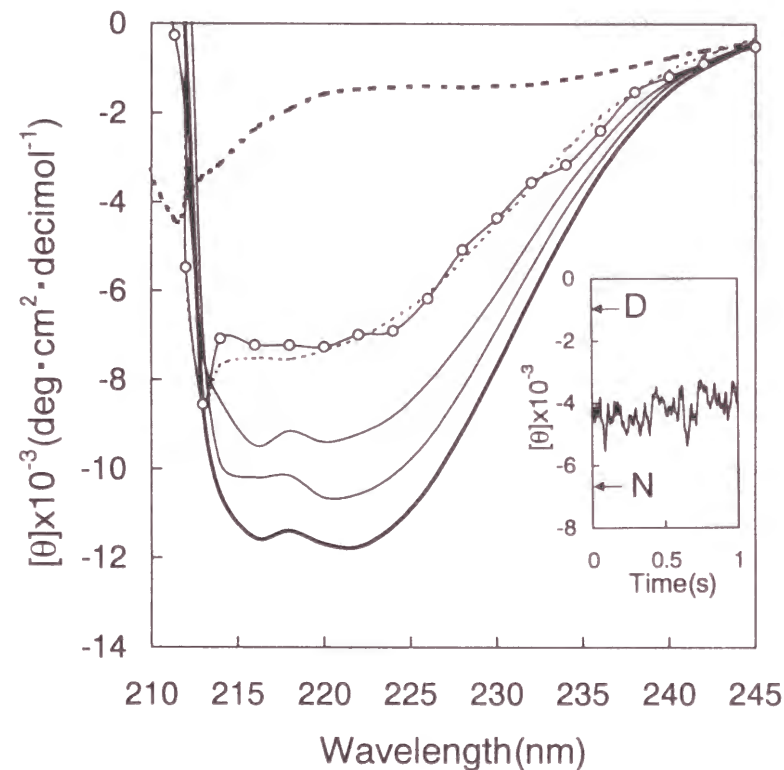


FIG. 7. Far-UV CD spectra of various states of ovalbumin.

The far-UV CD spectra of native ovalbumin (*thick solid line*), the protein denatured in 9 M urea (*thick broken line*), and the proteins refolded for 30 min and 20 h (*the upper and lower thin solid lines*, respectively) were recorded at 25°C as described in the text. The equilibrium intermediate state was produced by 20-fold dilution of the urea-denatured protein with 50 mM K-phosphate buffer, pH 2.2 containing 1.0 mM Na-EDTA and the CD spectrum was recorded in the same way (*thin dotted line*). For the CD spectra for early refolding intermediates, the time course of changes in CD ellipticities at different wavelength were recorded during the refolding and the values at the refolding time of 10 s were plotted as a function of wavelength (*open circles with thin solid line*). In the inset, the urea-denatured protein was refolded at pH 8.2 and the changes in CD ellipticities at 230 nm were recorded for one second using a stopped-flow instrument as described in the text. D and N shown by arrows represent the CD ellipticities at 230 nm of the urea denatured and native proteins, respectively.

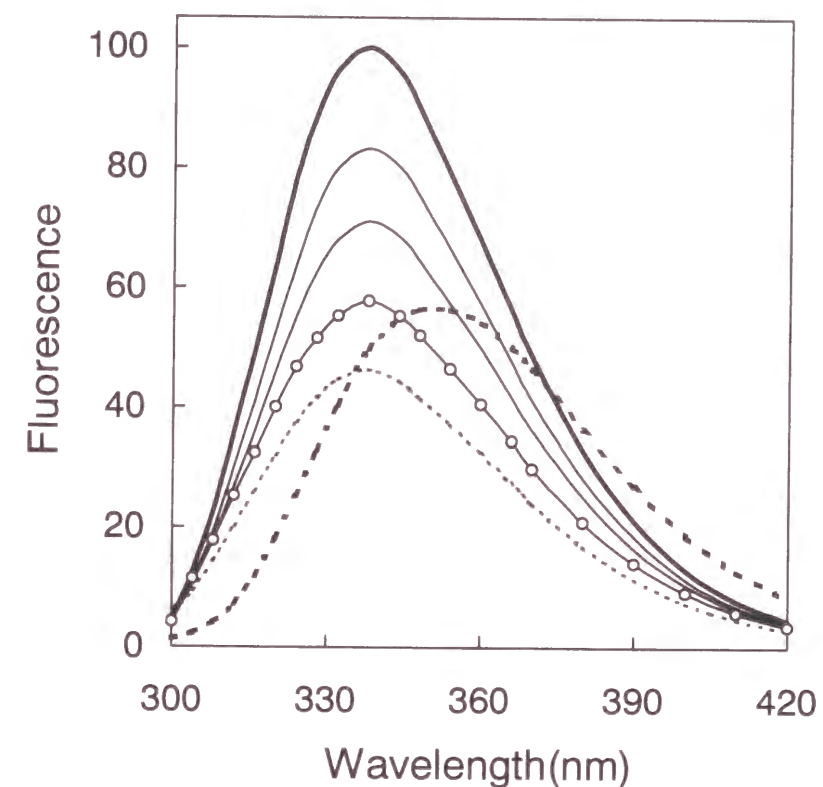


FIG. 8. Fluorescence emission spectra.

The tryptophan residues in native ovalbumin (*thick solid line*) and in the protein denatured in 9 M urea (*thick broken line*) were excited at 295 nm, and the emission spectra were recorded at 25°C. The intrinsic tryptophan fluorescence spectra of the protein refolded for 30 min and 20 h (*lower and upper thin solid lines*, respectively) were recorded in the same way. The thin dotted line represents the fluorescence spectrum for the equilibrium intermediate state that was produced by 20-fold dilution of the urea-denatured protein with 50 mM K-phosphate buffer, pH 2.2 containing 1.0 mM Na-EDTA. For the fluorescence spectra at an early refolding time, the time course of fluorescence emission changes were recorded during the refolding at different emission wavelengths and the values at refolding time 10 s were plotted as a function of emission wavelength (*open circles with thin solid line*). The fluorescence intensity is shown in arbitrary units.

Figs. 7 and 8 also show that after the initial burst phase, the absolute CD ellipticity and fluorescence intensity then increased slowly with increasing time of refolding. The protein refolded for a prolonged time of 20 h

showed 90% of the absolute CD ellipticity at 222 nm and 83% of the intrinsic fluorescence intensity at 338 nm of the native protein. The data from the two conformational analyses were therefore consistent with the formation of an initial burst intermediate having 60 % of the absolute CD ellipticity at 222 nm and 57% of the fluorescence intensity at 338 nm of the native form, during the refolding process.

The partially folded intermediate showing the same far-UV CD spectrum as the intermediate formed at pH 8.2 was also formed by dilution of the urea-denatured protein with an acidic buffer, pH 2.2 (Fig. 7). Under the same conditions, the fluorescence spectrum of ovalbumin showed the maximum at 338 nm that is consistent with the peak wavelength for the intermediate formed at pH 8.2 (Fig. 8). The decreased fluorescence intensity for the acid-quenched intermediate can be accounted for by solvent effects owing to acid (23,40). These spectral profiles were almost exactly the same as those of the previously observed intermediates formed from the acid/urea-denatured protein (40).

Time Course for the Refolding after the Initial Burst Phase

In a previous report (21), we have shown that a trypsin resistance assay is a sensitive probe for the analysis of the native conformation of ovalbumin. The trypsin resistance of different conformational states of ovalbumin was examined in more detail. As shown in Fig. 9, native ovalbumin was resistant to trypsin at protease concentrations up to 125 mg/ml. Almost all of the urea-denatured protein, however, was degraded

at a trypsin concentration as low as 12.5 mg/ml. Likewise, the acid-quenched intermediate that had structural characteristics equivalent to the initial burst refolding intermediate was almost completely degraded at the trypsin concentration of 12.5 mg/ml. This protease-sensitive nature should not be accounted for by the pH-jump procedure for the analysis of the acid-quenched intermediate, since native ovalbumin that had been pre-incubated at pH 2.2 showed protease resistance at 125 mg/ml of trypsin. The data in Fig. 9 therefore imply that the native protein, but not the urea-denatured or initial burst intermediate, is detected as the resistant molecule at a protease concentration higher than 12.5 mg/ml.

The time course for the refolding was examined by means of the trypsin resistance assay and the data were compared with the time courses, after the initial burst phase, obtained by the far-UV CD and intrinsic fluorescence analyses. Fig. 10 clearly demonstrates that the time course for the refolding was almost exactly the same for the three conformational probes. The refolding data were fitted well to a biphasic rate equation (the sum of two exponentials). Such biphasic kinetics is consistent with the involvement of disulfide rearrangements during the refolding (see DISCUSSION).

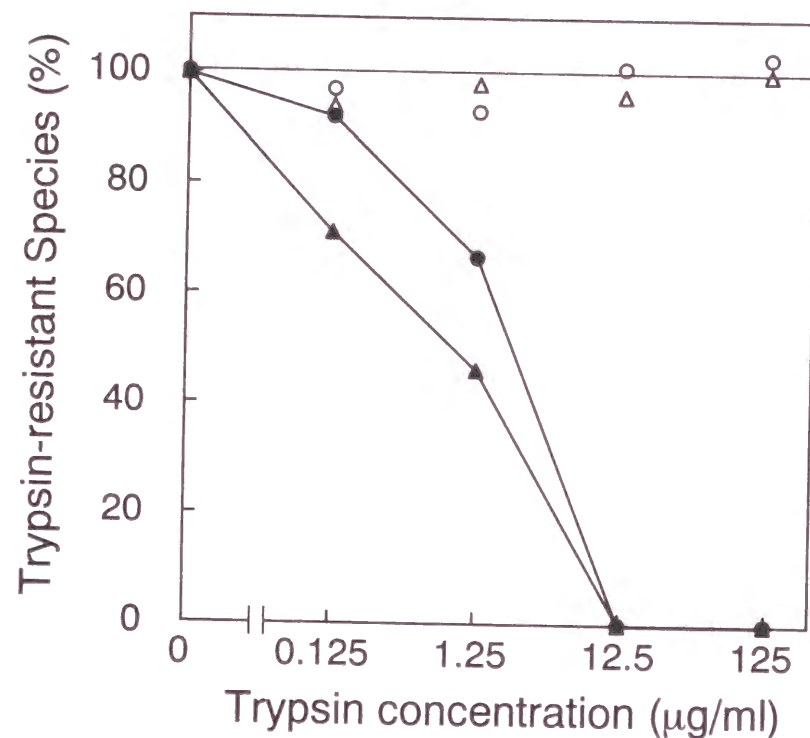


FIG. 9. Trypsin resistance of various states of ovalbumin.

Native ovalbumin dissolved at 1.0 mg/ml in buffer A (*open circles*) or in 50 mM K-phosphate buffer, pH 2.2 containing 1.0 mM Na-EDTA (*open triangles*), or the protein denatured in buffer A containing 9 M urea (*closed circles*) was diluted 20-fold with buffer A containing different concentrations of trypsin to give the final protease concentrations shown on the abscissa. After 1 min of incubation at 25°C, proteolysis was terminated by addition of soybean trypsin inhibitor. The samples were analyzed for sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the amounts of intact ovalbumin were estimated as described in the text. The equilibrium intermediate (*closed triangles*) that had been produced by 20-fold dilution of the urea-denatured protein with 50 mM K-phosphate buffer, pH 2.2 containing 1.0 mM Na-EDTA was also analyzed in the same way. The ordinate represents the amounts of the intact proteins at various protease concentrations, expressed as percentages of the values obtained without added protease.

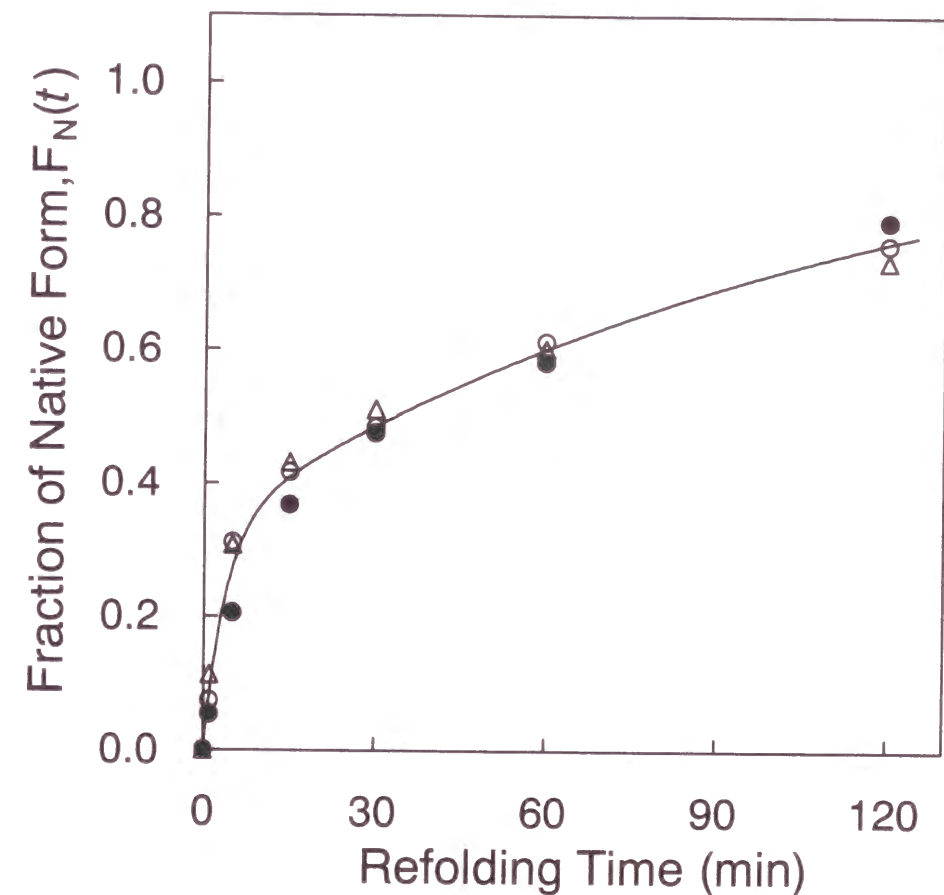


FIG.10. Time course for refolding after the initial burst phase.

The urea-denatured protein was refolded at pH 8.2, 25°C, and the time-dependent conformational regain was monitored in terms of the CD ellipticity at 222 nm (*closed circles*), the intrinsic tryptophan fluorescence at 338 nm (*open triangles*) and the trypsin resistance (*open circles*) as described in the text. The ordinate shown by $F_N(t)$ represents the fraction of the native form at the refolding time of t , calculated by using the equation: $F_N(t) = (X_0 - X_t) / (X_0 - X_N)$, where X_0 and X_t are the initial values and the values at the refolding times of t , respectively. For the CD and fluorescence analyses, the values at 6 s refolding were taken as X_0 . X_N is the final value of the refolding. The values at the refolding time of 20 h were employed as X_N ; the X_N values were 90, 83, and 80 % of the native values for the CD ellipticity, tryptophan fluorescence, and trypsin resistance analyses, respectively. The solid curves represent nonlinear least-squares fits of the experimental data to a biphasic rate equation:

$F_N(t) = A_1 + A_2 e^{-k_1 t} + A_3 e^{-k_2 t}$. Obtained constants were: 1.008 for A_1 , -0.670 for A_2 , -0.340 for A_3 , 0.00831 for k_1 , and 0.261 for k_2 .

Sulfhydryl/disulfide Exchanges during Refolding

The preceding data demonstrate the involvement of an initial burst intermediate in the refolding process of the urea-denatured ovalbumin. The author's previous study has demonstrated that ovalbumin undergoes, in the initial burst intermediate, disulfide rearrangements via intrachain sulfhydryl/disulfide exchange reactions (see Scheme 1 in chapter I). These data suggest that non-native disulfide isomers included in the urea-denatured ovalbumin are refolded into the native form with the correct disulfide bond through disulfide rearrangements.

To investigate this possibility, the author determined the disulfide-involved half-cystines by means of the peptide mapping procedure at various refolding times. As shown in Fig. 11, any of the six cysteine residues was found to be involved in a disulfide bond at the refolding time 0. Such a random distribution in the denatured protein rearranged in a slow reaction to a less random state. For Cys73 and Cys120 that form the native disulfide, the disulfide-involved amounts were only 20% at refolding time 0, but they increased with time of refolding. More than 70% of these cysteines participated in disulfides at a prolonged refolding time of 20 h. In contrast, for the other four cysteines the disulfide-involved amounts decreased with time of refolding. During the refolding, the number of free cysteine sulfhydryls was almost constant; the values were from 3.7 to 3.8, being essentially consistent with the number of free cysteine sulfhydryls in native ovalbumin. These data are consistent with the view that most, if not all, of the denatured non-native disulfide isomers can

refold through intrachain sulfhydryl/disulfide exchange reactions into the native disulfide form.

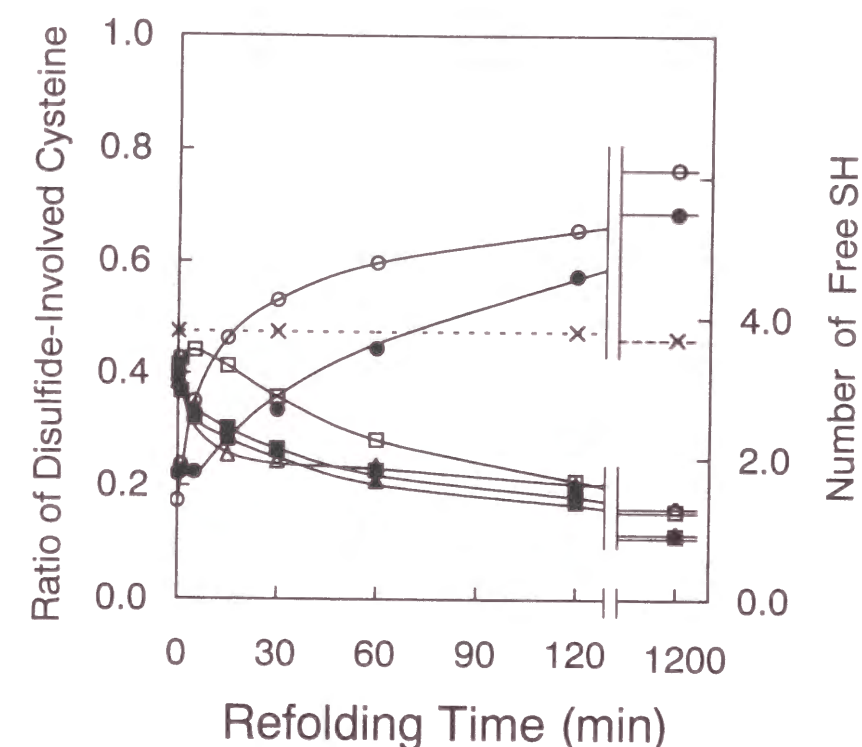


FIG. 11. Disulfide rearrangements during refolding.

At various times of refolding from the urea-denatured state, disulfide-involved cysteines for Cys11 (*open squares*), Cys30 (*closed squares*), Cys73 (*closed circles*), Cys120 (*open circles*), Cys367 (*closed triangles*), and Cys382 (*open triangles*) were determined using a peptide mapping analysis as described in the text. The number of free cysteine sulfhydryls (*cross*) was determined by amino acid analysis.

Analysis of the Integrity of the Refolding by Differential Scanning Calorimetry

The preceding data show that most, if not all, of the ovalbumin molecules refold into the native state within 20 h incubation (Figs. 10 and 11). The integrity of the refolding was also investigated by an alternative method of differential scanning calorimetry. As demonstrated in Figure 12, the protein refolded for 20 h showed a major thermal transition peak at 77.6°C, although a minor peak with lower melting temperature was also detected. The major transition temperature was almost exactly the same as the value for native ovalbumin (77.7°C).

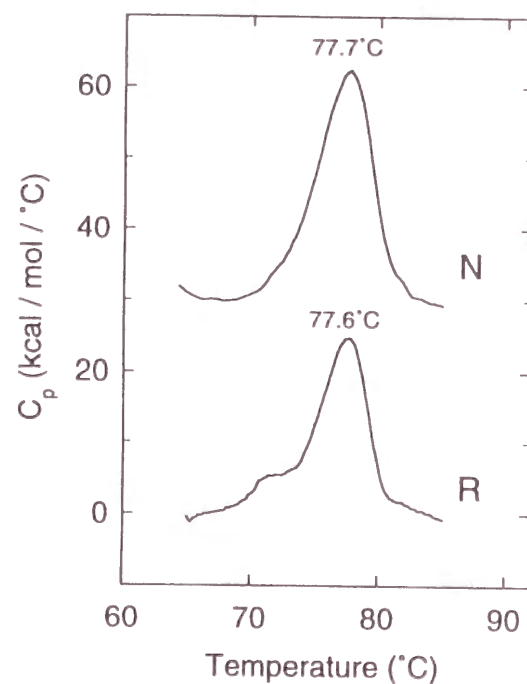


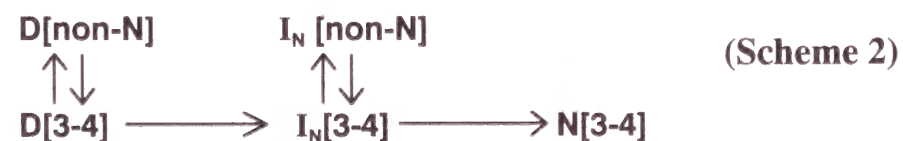
FIG. 12. Differential scanning calorimetry analysis of the refolded ovalbumin. Ovalbumin refolded for 20 h and native protein as a control were analyzed by differential scanning calorimetry in 10 mM Na-phosphate buffer, pH 6.0 with a temperature scanning rate of 1 K·min⁻¹. Endothermic transition profiles for the native protein (N) and for the refolded protein (R) are arbitrarily shifted on the ordinate scale for clarity.

DISCUSSION

In chapter I, the author investigated the refolding mechanism of ovalbumin using the acid/urea-denatured protein (in 9 M urea, pH 2.2) as the starting protein sample, since possible sulfhydryl/disulfide exchange reactions in urea-denatured ovalbumin are almost completely blocked (5). Refolding was initiated by a pH-jump procedure in which the acid/urea-denatured protein is placed in a refolding buffer of near-neutral pH (pH 8.2). It has been demonstrated using this refolding system that most of the denatured ovalbumin molecules can correctly refold via non-productive disulfide rearrangements in an initial burst intermediate I_N (see Scheme 1 in chapter I). In this chapter, ovalbumin was denatured at pH 8.2 in 9 M urea as the starting protein sample and then allowed to refold at the same pH value. Since the isoelectric point of A₁-ovalbumin is 4.58 (42), the electrostatic interactions should be quite different in the urea-denatured conditions at the two different pH values. The author have, however, observed that ovalbumin is in a random coil state in the presence of a high concentration of urea either at pH 8.2 (20) or at pH 2.2 (Tatsumi, E. and Hirose, M., unpublished observation). This indicates that both systems allow examination of the refolding process from random coil state to native state. The major difference in the two refolding systems is in the disulfide structures of the urea-denatured proteins: in a high concentration of urea at a near-neutral pH, but not at a strongly acidic pH, ovalbumin has been shown to undergo extensive disulfide rearrangements generating many disulfide isomers including the native disulfide isomer D[3-4] and

mispaired disulfide isomers D[non-N] that all contain one disulfide and four sulfhydryls in the molecule (20).

The results of optical and trypsin-resistance analyses in the present report demonstrate that most, if not all, of the urea-denatured D[3-4] and D[non-N] can refold into the native state (Fig. 10). In addition, the differential scanning calorimetry analysis revealed almost exactly the same denaturation temperature for the refolded and native proteins, although a minor shoulder peak with a lower denaturation temperature was detected for the former protein form (Fig. 12). During the refolding, a partially folded intermediate state was formed in the initial burst phase (Figs. 2 and 3); the far-UV CD and intrinsic tryptophan fluorescence spectra were almost exactly the same as those of the early intermediate state formed from the acid/urea-denatured protein (40). After the initial burst phase, regain of the native disulfide via disulfide rearrangements was observed during the refolding, in which the number of free cysteine sulfhydryls was almost constant (Fig. 11). These data were consistent with refolding of ovalbumin according to an extended version of Scheme 1:



where $\text{I}_N[\text{3-4}]$ and $\text{I}_N[\text{non-N}]$ are the initial burst refolding intermediate with the native disulfide and with a mispaired disulfide, respectively; the former is the competent intermediate for subsequent folding into the native form,

N[3-4] . Through disulfide rearrangements, $\text{I}_N[\text{3-4}]$ undergoes reversible interconversion with $\text{I}_N[\text{non-N}]$, and D[3-4] with D[non-N].

The data for the conformational regain after the initial burst phase fitted well to an equation consisting of the sum of two exponentials (see the legend of Fig. 10). The apparent rate constants obtained by the data fitting analysis (0.00831 for k_1 , and 0.261 for k_2) should include both the first-order rate constants for the disulfide rearrangements and for the folding from I_N to N[3-4] (40). Although, because of the lack for the initial values for D[3-4] and D[non-N], the rate constants for disulfide rearrangement and folding reactions could not be determined using a more sophisticated rate equation (40), the data in Fig. 10 clearly demonstrate that the time course for the conformational regain followed biphasic kinetics. As a mechanism for biphasic refolding kinetics, the involvement of the parallel pathway that is related to cis-trans isomerization of proline residues has been demonstrated (28). Ovalbumin contains 14 proline residues (18). As shown in a previous report (40), however, the biphasic refolding kinetics of disulfide-bonded ovalbumin can be accounted for by the involvement of disulfide rearrangements rather than by proline isomerization, since the disulfide-reduced ovalbumin refolds with simple monophasic kinetics.

The author's attempts to separate different disulfide isomers by ion exchange or reversed phase HPLC have all been unsuccessful, probably because of the large size of ovalbumin. This has made it difficult to determine in detail the pathway of the disulfide rearrangements during the

refolding. Scheme 2, however, indicates that a protein that contains a cystine disulfide along with cysteine sulfhydryls in the molecule may generally be a useful model for the investigation of protein folding mechanisms. First, if an anaerobic condition is employed, the numbers of intrachain disulfide and sulfhydryls are the same as those in the native protein during the refolding in which disulfide rearrangements are included (Fig. 11). This indicates that non-native disulfide isomers can refold into the native disulfide form by intrachain sulfhydryl/disulfide exchange reactions without the help of a catalytic reagent or of an enzyme. A major problem encountered in oxidative refolding studies, related to protein sulfhydryl accessibility to an oxidizing agent, can therefore be circumvented in the sulfhydryl/disulfide protein. Second, the unfolded protein that is usually employed as the starting protein for subsequent refolding should consist of a vast number of conformational isomers. The possibility that different subsets of the conformational isomers refold at different rates can not be ruled out. A disulfide isomer that is produced in a sulfhydryl/disulfide protein under denaturing conditions corresponds to a subset of conformational isomers; the conformational entropy of an isomer depends on the number of amino acid residues separating the two half-cystine residues (20, 43, 44). If the refolding mechanisms are compared for different disulfide isomers, the refolding pathway may be directly related to the free energies of the original denatured states.

CHAPTER III

Characterization of Refolding Intermediate of Ovalbumin Using Non-Native Disulfide Isomers

In chapter II, the author reported that urea-denatured ovalbumin comprising non-native disulfide isomers refold to native state and most, if not all, of the D[non-N] species can correctly refold into the native form via disulfide rearrangements according to Scheme 2. A clear refolding mechanism, however, seems difficult to get by using the mixture of disulfide isomers with an unknown pair of disulfide as the starting sample. In a previous study, a preparation method for urea denatured ovalbumin with the only disulfide Cys 367- Cys 382 (express by D[5-6]) has been established by using a chemical modification technique (46). In this chapter, the author more clearly investigates refolding from non-native disulfide species by use of D[5-6].

While Scheme 2 in chapter II denotes that any disulfide isomers of ovalbumin must undergo $I_N[3-4]$ via sulfhydryl/disulfide exchange reactions to refold from urea-denatured state to native state. We also established the method for preparation of another chemical modified ovalbumin which contains one non-native disulfide Cys 367-Cys 382, three cysteine sulfhydryls (Cys 11, Cys 30 and Cys 120) and carboxymethylated Cys 73 (express by D[3CM/5-6]), whose disulfide cannot rearrange to the native form Cys 73-Cys 120 on account of the blocked Cys 73. D[3CM/5-6] cannot refold to native state and stops refolding at I_N state. Utilizing, the unique structural situations of D[3CM/5-6], the author

concludes that non-native disulfide prevents I_N from refolding to native state, and characterized whole conformation of I_N as very compact near the native state.

EXPERIMENTAL PROCEDURES

Materials

A₁-ovalbumin was purified as described in former chapter. Diphenylcarbamyl chloride-treated trypsin (Type XI) and chymotrypsin (Type II) were purchased from Sigma. Achromobacter protease I (E.C. 3.4.21.50) was obtained from Wako Pure Chemical Industries.

Denaturation and Refolding of Ovalbumin

D[5-6] was prepared by chemical modification methods with 2,2'-dipyridyl disulfide as described elsewhere (46).

D[3CM/5-6] was prepared by the same method as preparation of D[5-6] except for the subsequent treatment for reducing. Reduced protein was incubating at 37°C for 10 min in buffer A (50 mM Tris-HCl buffer, pH 8.2, 1.0 mM Na-EDTA) containing 40 mM IAA in case of D[3CM/5-6].

D[3CM/SH] was prepared by incubating D[3CM/5-6] at ca 2.0 mg/ml, 37°C for 30 min in buffer B (9 M urea, 5 mM DTT, 50 mM Tris-HCl buffer, pH 8.2, 1.0 mM Na-EDTA). Buffer substituted 9 M urea-0.25 M HCl containing 1.0 mM Na-EDTA for buffer B through a Sephadex column (NAP-25, Pharmacia Biotech Inc.) after reducing treatment. D[SH] is the same as urea denatured proteins in SH condition described in chapter I.

Refolding was initiated at 25 °C by 20-fold dilution of urea-denatured proteins with buffer A giving a final pH value of 8.2. The proteins were allowed to refold at 25 °C and were then analyzed by trypsin resistance, intrinsic tryptophan fluorescence and CD spectrum. The buffers were degassed at reduced pressure and equilibrated under N₂ atmosphere prior to the refolding. An equilibrium intermediate I_A was produced by 20-fold dilution of urea-denatured proteins with 50 mM potassium phosphate buffer, pH 2.2, containing 1.0 mM Na-EDTA. For the experiments in the D[3CM/SH] and D[SH] condition, buffer A was containing 0.5mM dithiothreitol.

Measurement of Intrinsic Tryptophan Fluorescence and CD Spectrum

The fluorescence spectrum of ovalbumin was measured with a Hitachi fluorescence spectrophotometer (Model F-3000). The intrinsic tryptophan residues in ovalbumin were excited at 295 nm and the time course of fluorescence intensity change was monitored at 338 nm emission. The far-UV CD spectrum of ovalbumin was recorded with a spectropolarimeter (JASCO, J-720). All measurements were carried out at a constant temperature of 25°C. The manner of measurements and so forth were carried out as described in chapter I.

Stopped-flow Analysis

For rapid mixing experiments, stopped-flow reaction analyzer (Applied Photophysics Ltd. UK) was employed. Changes in the fluorescence

intensity at 338 nm of the urea-denatured ovalbumin excited at 295 nm were recorded at 25°C after 10-fold dilution with buffer A. The dead time for mixing was determined 4 ms by using the reaction between 2,6-dichlorophenolindophenol and L-ascorbate (25).

Trypsin-resistance Assay

Refolded ovalbumin with the native conformation is considered to be a trypsin resistant species as described (21). All procedure and analysis for assay were carried out as the same manner described chapter II.

Differential Scanning Calorimetry

The denatured protein was refolded for 20 h and concentrated about 15-fold using a concentrator (Amicon, Centriprep-10). The refolded protein and native protein control were analyzed with a differential scanning calorimeter (Micro Cal, MCS-DSC). The protein concentration was 0.5mg/ml in buffer A. The rate of temperature change was 1 K·min⁻¹.

Analyses for Disulfide-involved Half-cystines

At various refolding times, sulfhydryl/disulfide exchanges were quenched by mixing the protein samples with 0.24 volume of 2 M HCl. Disulfide-involved half-cystines were determined by alkylation with a fluorescent reagent IAEDANS, and subsequent peptide mapping procedure as described elsewhere (20).

Size Exclusion Chromatography Analysis

Blue dextran, sodium azide, A₁-ovalbumin and the following proteins were used for column calibration; bovine liver catalase (nacalai tesque), rabbit muscle lactate dehydrogenase (Sigma type XI), human transferrin (Sigma grade II), bovine serum albumin (Sigma), bovine erythrocyte carbonic anhydrase (nacalai tesque). HPLC were performed with a Shimazu UV absorbance monitor SPD-2AS (set at 220 nm). Analytical gel filtration experiments were performed by use of TSKgel G3000SW_{XL} column (7.8 mm I.D.×30 cm). Column temperature was maintained at 25°C. The flow rate through the column was maintained at 0.5 ml/min. Column was equilibrated with the following elution buffer: buffer A; for native state samples, refolding samples and column calibration, 50 mM K-phosphate buffer (pH2.2); for I_A samples, 9 M urea-0.25 M HCl; for urea-denatured samples. Native state samples or refolding samples were prepared by 20-fold dilution of 1mg/ml native or urea-denatured protein with buffer A, respectively. Urea-denatured samples and I_A samples were prepared by 20-fold dilution of 1mg/ml urea-denatured protein with 9 M urea- 0.25 M HCl or 50 mM K-phosphate buffer, respectively. Procedure of HPLC analysis and calculation of stokes radius were pursuant as described (47, 48).

ANS Binding Experiments

ANS in the presence of ovalbumin in various states were excited at 350 nm and emission spectra were recorded at the wavelength range from 370

nm to 610 nm with a fluorescence spectrophotometer (Hitachi, F-3000). All measurements were carried out at a constant temperature of 25 °C. Native state samples, refolding samples, urea-denatured samples and I_A samples were prepared as decried above. ANS spectra were recorded after 30 s adding 0.005 volume of 4.02 mM ANS to each sample, except for measurements in the 30 s-refolding sample. In case of the 30 s-refolding samples, urea-denatured protein were diluted with buffer A containing 21 μ M ANS. The time course of fluorescence intensity change was monitored at 470 nm emission. For the spectrum measurements at 30 s-refolding time, the time course of fluorescence intensity changes was monitored at various emission wavelengths with excitation at 350 nm, and data at a refolding time of 30 s were plotted. The final concentration of protein and ANS were 49.75 and 20 μ M, respectively.

RESULTS

Folding Intermediate Detected by Intrinsic Tryptophan Fluorescence and Far-UV CD Spectra

In former chapters, two types of partially folded intermediates I_N and I_A have been shown to be formed in an initial burst phase, as detected by far-UV CD and intrinsic tryptophan fluorescence spectra, from D[3-4], D[SH] and mixture of disulfide isomers D[mix]. In this chapter, the author has examined about two types of non-native disulfide species D[5-6] and D[3CM/5-6]. D[3CM/SH] which was prepared by reducing the disulfide Cys 367-Cys382 of D[3CM/5-6] also examined and compared with D[SH]

to inspect for the effect of carboxymethylated Cys 73. As shown in Fig. 13, the fluorescence emission spectrum of the native form had an emission maximum at 338 nm. The urea-denatured protein showed a typical red-shifted spectrum of an unfolded protein: the emission maximum was shifted to a longer wavelength of 352 nm, and the fluorescence intensity was decreased to 32% of the native form. When the urea-denatured ovalbumin was refolded, the protein showed at an early refolding time of 5 ms a fluorescence spectrum that had a peak at the same wavelength, but with much decreased intensity as compared with the spectrum of native ovalbumin. As shown in Fig. 14, the refolding proteins from the urea-denatured state showed at the early stage of 10 s an intermediate CD spectrum that had 60% of the absolute ellipticity at 222 nm of the native form. Figs. 13 and 14 also show that after the initial burst phase, the absolute CD ellipticity and fluorescence intensity then increased slowly with increasing time of refolding. The data from the two conformational analyses were therefore consistent with the formation of an initial burst intermediate having 60 % of the absolute CD ellipticity at 222 nm and 58% of the fluorescence intensity at 338 nm of the native form, during the refolding process.

The partially folded intermediate showing the same far-UV CD spectrum as the intermediate formed at pH 8.2 was also formed by dilution of the urea-denatured protein with an acidic buffer, pH 2.2 (Fig. 14). Under the same conditions, the fluorescence spectrum of ovalbumin showed the maximum at 338 nm that is consistent with the peak wavelength for the

intermediate formed at pH 8.2 (Fig. 13). These spectral profiles were almost exactly the same as those of the previously observed intermediates formed from the acid/urea-denatured protein (40). It confirmed that similarly the initial burst intermediates are also formed during the refolding from D[5-6], D[3CM/5-6] or D[3CM/SH].

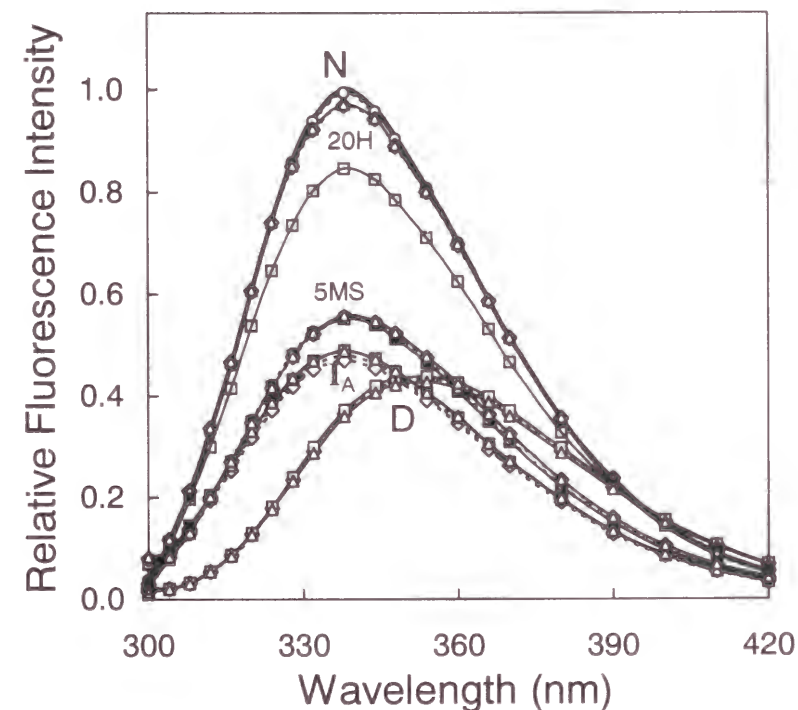


FIG.13. Fluorescence emission spectra of various states of ovalbumin.

Tryptophan residues in various states of ovalbumin were excited at 295 nm, and the emission spectra were recorded at 25 °C as described under "Experimental Procedures." N (*thick lines*) represents native ovalbumin. D is the urea-denatured protein as the following: D[5-6] (*open triangles with thin solid line*), D[3CM/5-6] (*open squares with thin solid line*), D[3CM/SH] (*open circles with thin dotted line*), D[SH] (*open diamonds with thin dotted line*). The equilibrium intermediate state I_A was produced by 20-fold dilution of D with a non-denaturing buffer, pH 2.2. The intrinsic tryptophan fluorescence of the proteins refolded from D for 20h(20H) was recorded in the same way. For the fluorescence spectra at 5ms(5MS), the time course of fluorescence changes was recorded during refolding from D at different emission wavelengths by use of a stopped flow analyzer (see Stopped-flow Analysis in Experimental Procedures), and the values at the refolding time of 5ms were plotted as a function of emission wavelength. The fluorescence intensity is shown by an arbitrary unit.

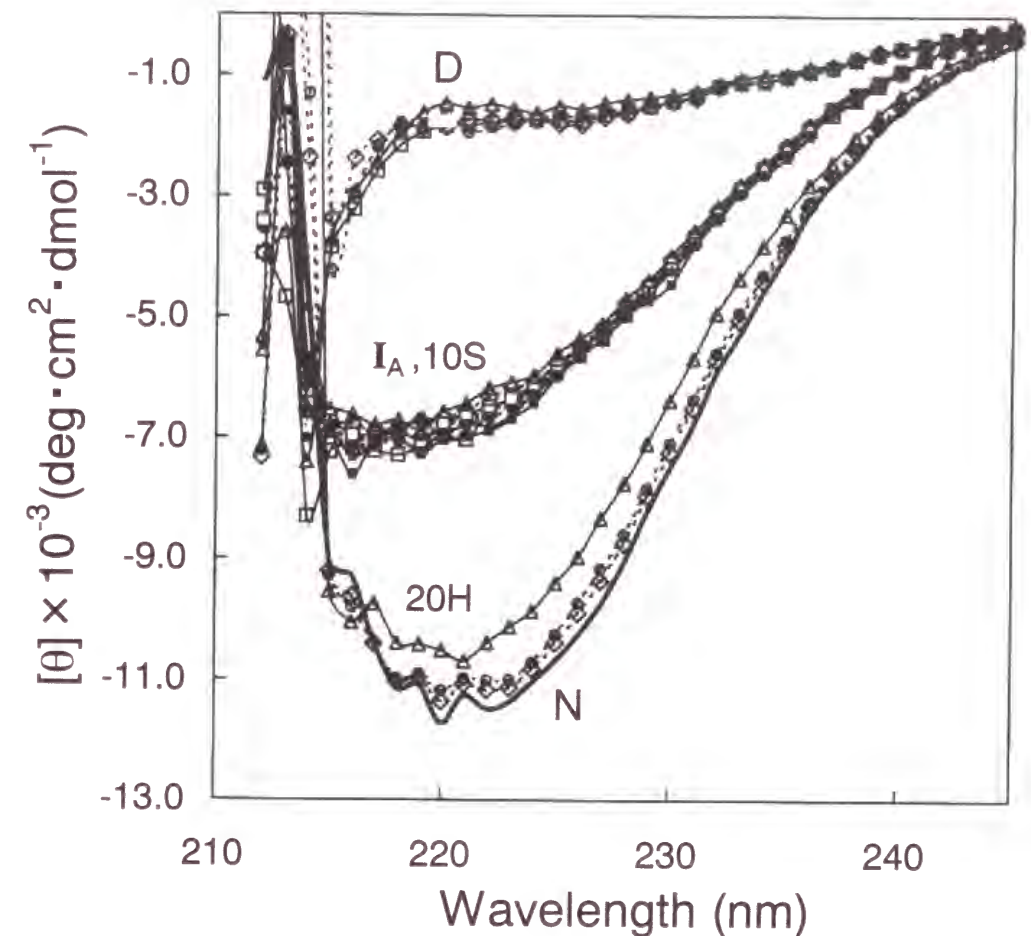


FIG.14. Far UV CD spectra.

The far UV CD spectra in various states of ovalbumin were recorded at 25 °C as described under "Experimental Procedures." The designations of N, D, I_A and 20H for the different ovalbumin states are the same as in Fig.13. For the CD spectra of refolding for 10s(10S, closed symbols), the time course of changes in CD ellipticities at different wavelengths were recorded during, and the values at the refolding time of 10 s were plotted as a function of wavelength.

Experiments for Detecting of Burst Phase by Stopped-flow Analysis.

Change of fluorescence intensity during the initial burst phase was measured by use of a stopped-flow reaction analyzer. As shown in Fig. 15, the obtained data was consistent with completion of the formation of

intermediate from each urea-denatured isomer within the mixing dead time of 4 ms. These results indicate that the initial burst phase proceeds regardless of the type of disulfide.

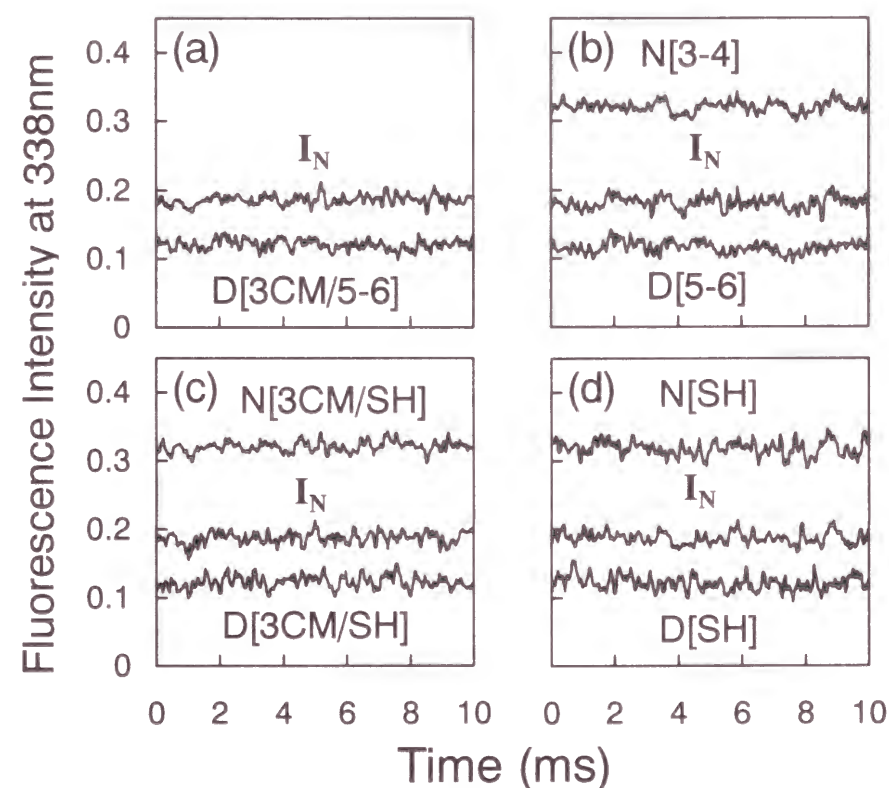


FIG.15. Stopped-flow analysis for the initial burst phase.

All proteins were excited at 295 nm, and the changes in fluorescence intensity at 338 nm were recorded for 10 ms using a stopped-flow analyzer as described under "Experimental Procedures". I_N is the refolded protein from each urea-denatured protein (D[3CM/5-6]; *a*, D[5-6]; *b*, D[3CM/SH]; *c*, D[SH]; *d*) at pH 8.2. N[3-4] and N[SH] are native proteins under the disulfide-bonded and reduced conditions (see chapter I). N[3CM/SH] was prepared by incubating 12mg/ml N[SH] with buffer A in the presence of 40mM IAA at 37°C for 10min (only Cys 73 is carboxymethylated completely under the conditions).

Time Course for the Refolding after the Initial Burst Phase

The time course for the refolding after the initial burst phase was examined by means of the trypsin resistance assay, far-UV CD and intrinsic fluorescence analyses (Fig. 16). It was almost exactly the same for the three conformational probes on refolding from D[5-6], D[3CM/SH] and D[SH]. The refolding data of D[5-6] was fitted well to a biphasic rate equation (the sum of two exponentials). Such biphasic kinetics are consistent with the involvement of disulfide rearrangements during the refolding. D[3CM/SH] refolded with simple monophasic first-order kinetics similarly to D[SH]. No significant effects of carboxymethylated Cys 73 were observed in the kinetic after the initial burst phase. There are the most important point to be noticed in the data of D[3CM/5-6]. Trypsin resistance and CD ellipticity did not recover in that. These results indicate that D[3CM/5-6] cannot make up its secondary structure after the initial burst phase and should not refold to the native state. The fluorescence intensity, however, partly recover in the data of D[3CM/5-6]. Although this data suggests that the intermediate I_N might be more stable after the initial burst phase, the author considers different factor (see DISCUSSION).

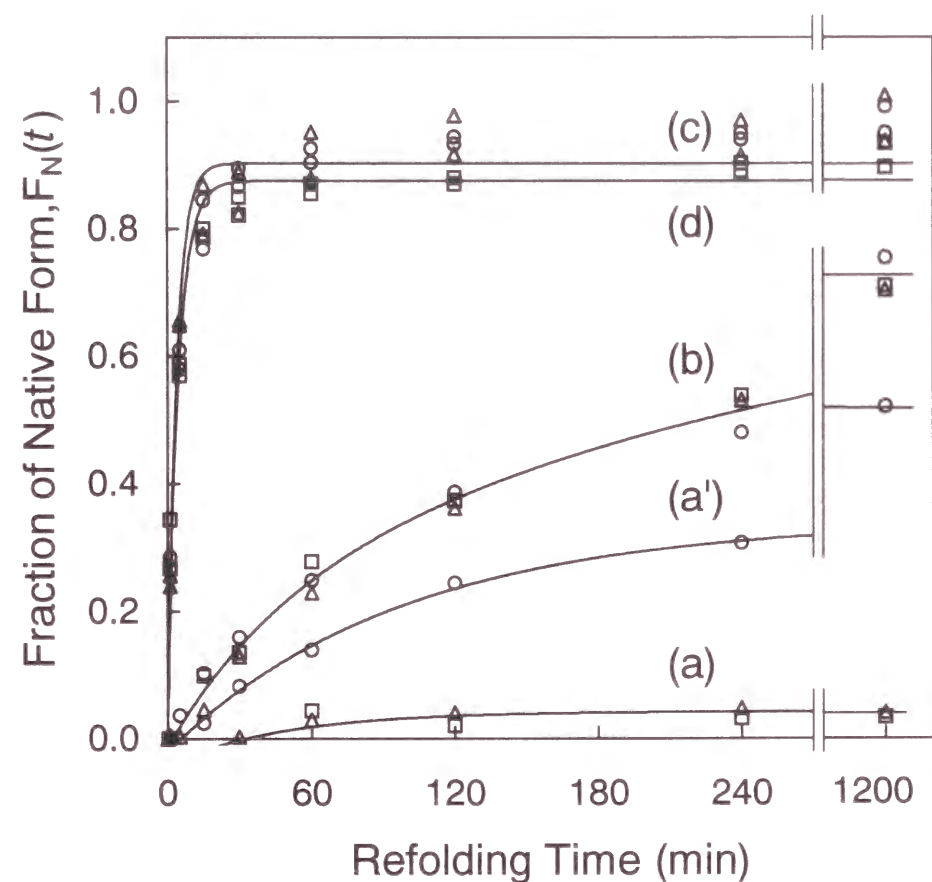


FIG.16. Time course for refolding after the initial burst phase.

The urea-denatured proteins (D[3CM/5-6]; *a* and *a'*, D[5-6]; *b*, D[3CM/SH]; *c*, D[SH]; *d*) was refolded at pH 8.2, 25°C, and the time-dependent conformational regain was monitored in terms of the CD ellipticity at 222 nm (*open triangles*), the intrinsic tryptophan fluorescence at 338 nm (*open circles*) and the trypsin resistance (*open squares*) as described in the text. The ordinate shown by $F_N(t)$ represents the fraction of the native form at the refolding time of t , calculated by using the equation: $F_N(t) = (X_0 - X_t) / (X_0 - X_N)$, where X_0 and X_t are the initial values and the values at the refolding times of t , respectively. For the CD and fluorescence analyses, the values at 6 s refolding were taken as X_0 . X_N is the final value of the refolding. The values at the refolding time of 20 h were employed as X_N ; the X_N values were 98, 95, and 95 % of the native values for the CD ellipticity, tryptophan fluorescence, and trypsin resistance analyses, respectively. The solid curves represent nonlinear least-squares fits of the experimental data to a monophasic rate equation: $F_N(t) = A_1 + A_2 e^{-k_1 t}$ (*a*, *c* and *d*) or biphasic rate equation: $F_N(t) = A_1 + A_2 e^{-k_1 t} + A_3 e^{-k_2 t}$ (*a'* and *b*). Obtained constants were shown at Table 3.

Differential Scanning Calorimetry Analysis of Refolded Ovalbumin

Intrinsic tryptophan fluorescence and far-UV CD spectra showed that most, if not all, of ovalbumin molecules refold into the native state at 20 h incubation from D[5-6] and D[3CM/SH] (Figs. 13 and 14). The integrity of native refolding was investigated more rigorously by an alternative method of differential scanning calorimetry. Fig. 17 demonstrates that the protein refolded from D[5-6] at 20 h incubation underwent thermal transition with almost the same melting temperature as did the native protein counterpart; the thermal denaturation temperatures were 77.7 °C for the native protein and 77.2 °C for the refolded protein from D[5-6]. No difference was detected in the melting temperature among the disulfide reduced protein N[SH], N[3CM/SH], refolded protein from D[SH] and refolded protein from D[3CM/SH]. These results indicate that disulfide reduced ovalbumin, whether whose Cys 73 is carboxymethylated or not, can refold correctly into the same protein energy states as the native proteins. At the data of refolded protein from D[3CM/5-6], a peak was almost undetectable. This data concludes that refolded protein from D[3CM/5-6] does not refold to the stable molecule like the native state and stops refolding at the unstable intermediate. A slight bulge in the data may be account for the minor contamination from D[3CM/SH] which generates during the preparation of D[3CM/5-6] and is contained 7% lower.

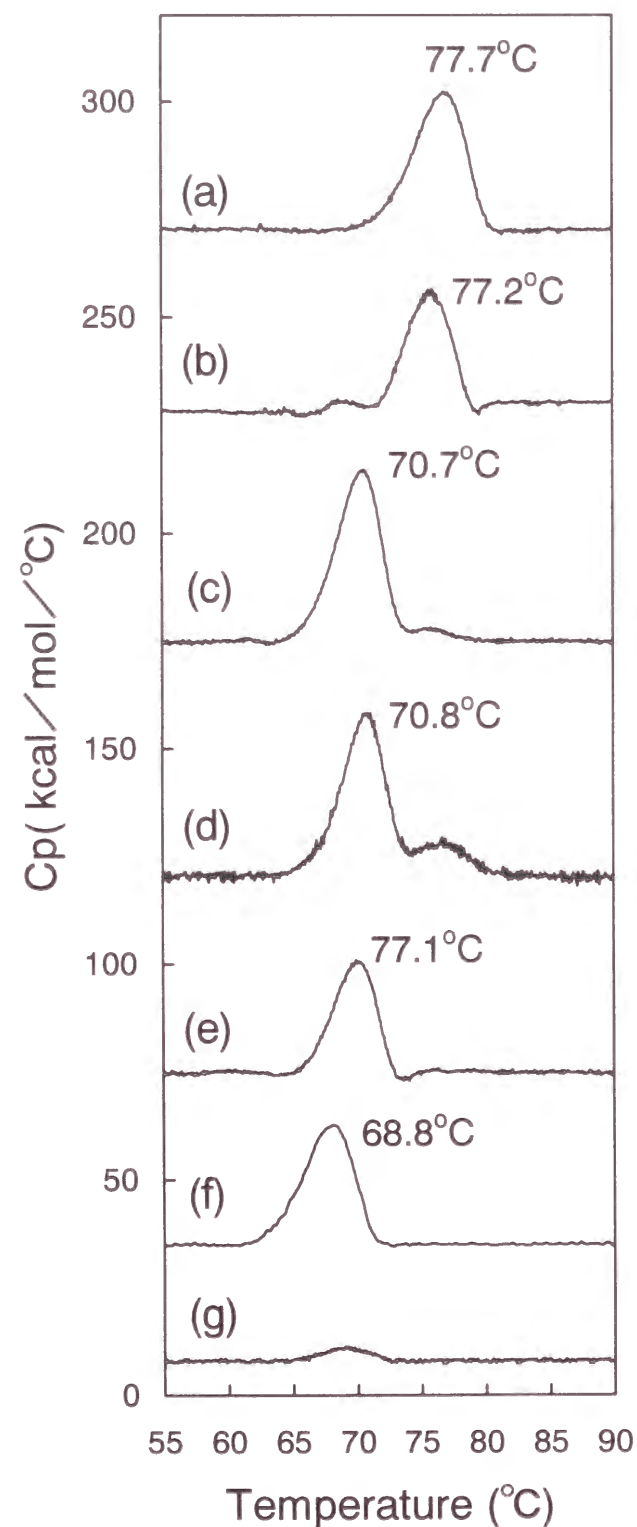


FIG.17. Differential scanning calorimetry analysis of refolded ovalbumin.

Refolded protein from urea-denatured state (D[5-6]; b, D[SH]; d, D[3CM/SH]; f, D[3CM/5-6]; g) at 20 h incubation and native protein (N[3-4]; a, N[SH]; c, N[3CM/SH]; e, these are the same as in Fig.15) controls were analyzed by differential scanning calorimetry in buffer A, with a temperature scanning rate of $1 \text{ K} \cdot \text{min}^{-1}$. Endothermic transition profiles are arbitrarily shifted on the *ordinate* scale for clarity.

Sulfhydryl/Disulfide Exchanges during Refolding

The previous my studies (40, 49) have demonstrated that ovalbumin undergoes, in the initial burst intermediate, disulfide rearrangements via intrachain sulfhydryl/disulfide exchange reactions. The fact leads to the suggestion that urea-denatured ovalbumin with a non-native disulfide can refold into the native form with rearrangement of disulfide to the native pair. To investigate this possibility, the author determined the disulfide-involved half-cystines during refolding from D[5-6] and D[3CM/5-6] by means of the peptide-mapping procedure at various refolding times. As shown in Fig. 18A, where expresses the data of refolding from D[5-6], only Cys 367 and Cys 382 were detected as disulfide-involved cysteines at the refolding time 0. The disulfide-involved Cys 367 and Cys 382 decreased immediately after the beginning of refolding ; concomitantly at this stage, all of the other four cysteines Cys 11 , Cys 30, Cys 73 and Cys 120 were detected as the disulfide-involved cysteines. The disulfide-involved Cys 73 and Cys 120, however, both increased gradually and the amounts were about 80% at 20 h refolding. In contrast, for the other four cysteines the disulfide-involved amounts decreased with time of refolding. These data are consistent with the view that most, if not all, of urea-denatured ovalbumin with a non-native disulfide can refold to the native disulfide form by intrachain sulfhydryl/disulfide exchange reactions without the help of a catalytic reagent or of an enzyme. Moreover, these results suggest that the refolding intermediate I_N is so flexible as to rearrange its disulfide with easy because the disulfide exchange reactions, at the least, occur twice to

change from Cys 367-Cys 382 to Cys 73-Cys 120.

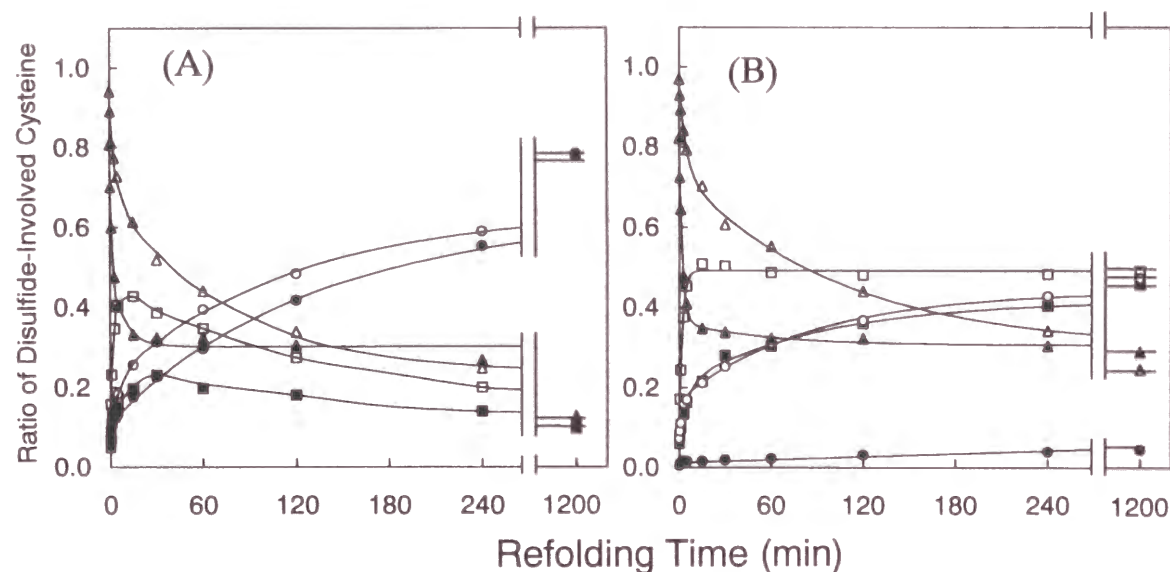


FIG.18. Disulfide rearrangements during the refolding.

D[5-6] (in panel A) and D[3CM/5-6] (in panel B) were refolded at pH 8.2, 25 °C. At various refolding times, the disulfide-involved cysteines for Cys 11 (open squares), Cys 30 (closed squares), Cys 73 (closed circles), Cys 120 (open circles), Cys 367 (open triangles), and Cys 382 (closed triangles) were determined using a peptide mapping analysis (20). The data are the averages for duplicate determinations. The solid curves represent linear or nonlinear least-squares fits of the experimental data: $f(t) = A_1 + A_2 t$ (Cys 73 in panel B), $f(t) = A_1 + A_2 e^{-k't}$ (Cys 11 in panel B), $f(t) = A_1 + A_2 e^{-k't} + A_3 e^{-k''t}$ (Cys 73 in panel A, Cys 30 in panel B and Cys120, Cys 367, Cys 382 in both panels). Obtained constants were shown at Table 3. The solid curves of Cys 11 and Cys 30 in panel A were obtained by curves smoothing between the data.

The data in Fig. 18B, where expresses the data of refolding from D[3CM/5-6], also showed only Cys 367 and Cys 382 were detected as disulfide-involved cysteines at the refolding time 0 and decreased after the beginning of refolding concomitantly increasing of Cys 11, Cys 30 and Cys 120. Cys 73, of course, did not increased during refolding because of blocked sulfhydryl; as a result, the disulfide did not exchange to native

form Cys 73-Cys 120. Intrachain sulfhydryl/disulfide exchange reactions continued in this protein at 20 h because various disulfide isomers had come to equilibrium as a mixture. The amounts of disulfide-involved Cys 11, Cys 30 and 120 were about 47%, and Cys 367 and Cys 382 were about 27% at 20 h refolding respectively. These data indicate that ovalbumin whose disulfide cannot exchange to native form does not misfold to a particular non-native disulfide molecule but stops a flexible intermediate with sulfhydryl/disulfide exchange reactions after the initial burst phase. Furthermore, the amount of disulfide-involved Cys 382 decreased more rapidly than Cys 367, and rapid increasing of Cys 11 occurred concomitantly. The data suggest that conformation of the flexible intermediate may be compact like the native form. The distance of Cys 11-Cys 367 is much further than that of Cys 367-Cys 382 on primary structure but is the most approximate on tertiary structure of native ovalbumin expect for Cys 73-Cys 120 (Fig. 6 in chapter II).

Size Exclusion Chromatography Analysis in Various State of Ovalbumin

The author experimented size exclusion chromatography analysis by taking advantage of the refolded protein from D[3CM/5-6] as the initial intermediate I_N and the diluted urea-denatured protein with acidic buffer as the stable intermediate I_A . As shown Table 2, the disulfide type of molecule hardly affected stokes radius in the native (30.3-31.0 Å), I_A (38.6-39.4 Å) or urea-denatured (58.2-59.5 Å) states. Refolded protein from D[3CM/5-6] was compact (31.6-32.5 Å) near the native protein rather than

the another intermediate I_A or extended urea-denatured protein at any refolding time.

TABLE 2

Stokes radius of ovalbumin in various states.

Stokes radius was calculated by use of size exclusion chromatography analysis as described under "Experimental Procedures". Native protein N[3-4], N[SH] and N[3CM/SH] are the same as in Fig. 15.

Samples	Stokes radius (Å)
N[3-4]	30.3±0.06
N[SH]	30.7±0.12
N[3CM/SH]	31.0±0.10
Refolding from D[3CM/5-6] for 16min	32.5±0.18
Refolding from D[3CM/5-6] for 120min	31.8±0.18
Refolding from D[3CM/5-6] for 20h	31.6±0.15
I_A [3-4]	38.6±0.21
I_A [SH]	39.0±0.09
I_A [3CM/SH]	39.4±0.15
I_A [5-6]	39.0±0.12
I_A [3CM/5-6]	39.2±0.17
D[3-4]	58.2±0.17
D[SH]	59.1±0.12
D[3CM/SH]	59.5±0.06
D[5-6]	58.6±0.21
D[3CM/5-6]	58.5±0.19

distinguishable from another intermediate I_A conformationally. ANS binding analysis more clearly indicated the distinction between two intermediates. Fig. 19 shows the emission spectra of ANS in the presence of ovalbumin in various states. In the native and urea-denatured state, ovalbumin showed no detectable ANS binding. In contrast, greatly increased fluorescence emission with a peak at 472nm was observed in the presence of intermediate I_A with any disulfide. The protein refolded from urea-denatured state by diluting with buffer A showed low level of ANS binding even 30 s-refolding. The decreasing of ANS binding was, concomitantly refolding, detected and consistent with time course for the refolding after the initial burst phase (Figs. 16, 20 and Table 3); refolded protein from D[3CM/5-6] kept even level of ANS binding for 20 h. These results indicate that the intermediate I_A has exposed hydrophobic core, and the intermediate I_N has closed conformation.

ANS Binding Analysis

The preceding study has demonstrated that the initial intermediate I_N is, in spite of flexible molecule, very compact near the native form and clearly

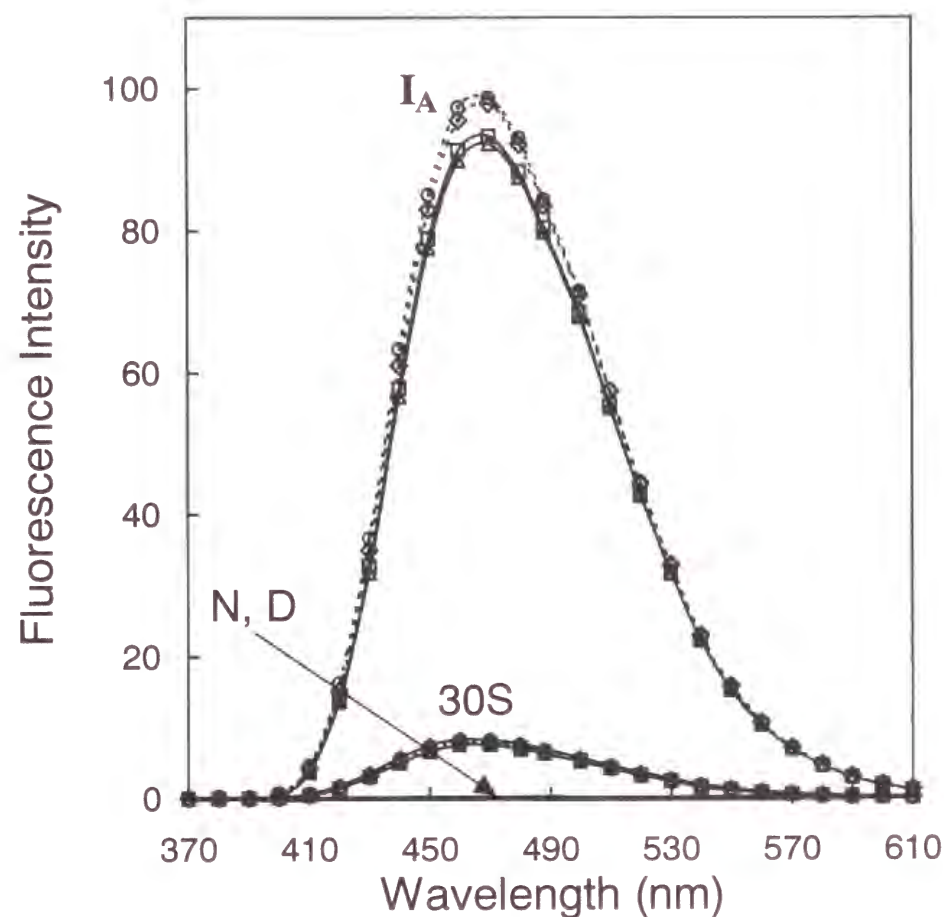


FIG.19. ANS binding to various states ovalbumin.

ANS in the presence of ovalbumin in various states were excited at 350 nm and emission spectra were recorded. The fluorescence intensity was not detectable in the presence of any native samples or urea-denatured samples (shown by *N, D* with an arrow). I_A and 30S represent the spectra in the presence of I_A samples and 30 s-refolding samples, respectively from D[5-6] (open triangles with thin solid line), D[3CM/5-6] (open squares with thin solid line), D[3CM/SH] (open circles with thin dotted line), D[SH] (open diamonds with thin dotted line). ANS spectra were recorded after 30 s adding 0.005 volume of 4.02 mM ANS to each sample, except for the measurements in 30 s-refolding sample. In case of 30s-refolding sample, urea-denatured protein were diluted with buffer A containing 21 μ M ANS. The final concentration of ANS was 20 μ M.

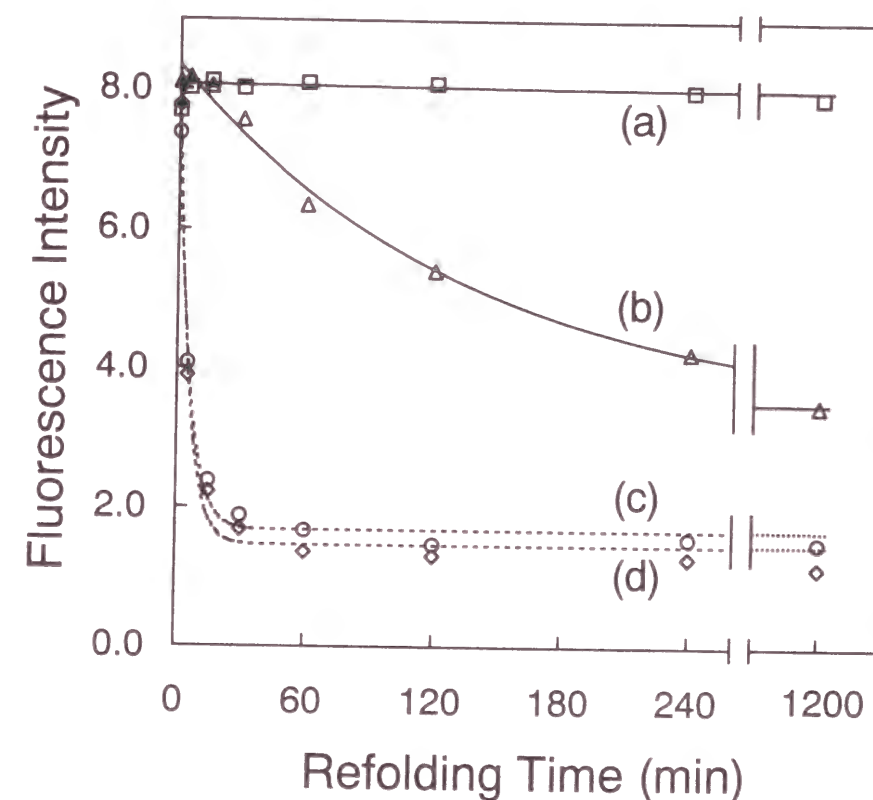


FIG.20. Time course of ANS binding during refolding.

The urea-denatured protein (D[3CM/5-6]; *a*, D[5-6]; *b*, D[3CM/SH]; *c*, D[SH]; *d*) was refolded at pH 8.2, 25°C, and the time-dependent conformational regain was monitored in terms of ANS binding. ANS was excited at 350 nm, and fluorescence intensity was monitored at 470 nm emission after 30 s adding 0.005 volume of 4.02 mM ANS to refolding samples at various refolding time. Only in the measurements of 30 s-refolding sample, urea-denatured protein were diluted with buffer A containing 21 μ M ANS. The final concentration of ANS was 20 μ M. The solid curves represent linear or nonlinear least-squares fits of the experimental data: $f(t) = A_1 + A_2 t$ (*a*), $f(t) = A_1 + A_2 e^{-k_1 t}$ (*c* and *d*), $f(t) = A_1 + A_2 e^{-k_1 t} + A_3 e^{-k_2 t}$ (*b*). Obtained constants were shown at Table 3.

TABLE 3

The constants obtained from least-squares fitting.

Many kinetic parameters were obtained from various time course experiments (A) and peptide mapping analysis (B) mentioned above (see Figs 16, 18 and 20). ^a FL, CD, TR and ANS represent the fluorescence, CD ellipticity, trypsin resistance and ANS binding analysis, respectively. ^bEquations used fitting are as follows: Eq.I; $f(t) = A_1 + A_2 e^{-k_1 t}$, Eq.II; $A_1 + A_2 e^{-k_1 t} + A_3 e^{-k_2 t}$, Eq.III; $f(t) = A_1 + A_2 t$. ^cCysteine in B represents disulfide-involved cysteine.

(A)

Refolding from	Probe ^a	Equation ^b	Amplitudes			Rate constants (min ⁻¹)	
			A ₁	A ₂	A ₃	k ₁	k ₂
D[5-6]	FL+CD+TR	Eq.II	0.79	-0.57	-0.23	0.0031	0.021
D[5-6]	ANS	Eq.II	3.4	2.5	2.5	0.0074	0.0074
D[3CM/5-6]	FL	Eq.II	0.34	-0.18	-0.18	0.010	0.010
D[3CM/5-6]	CD+TR	Eq.I	0.042	-0.084		0.021	
D[3CM/5-6]	ANS	Eq.III	8.1	-0.0002			
D[SH]	FL+CD+TR	Eq.I	0.87	-0.80		0.21	
D[SH]	ANS	Eq.I	1.5	6.9		0.19	
D[3CM/SH]	FL+CD+TR	Eq.I	0.91	-0.81		0.21	
D[3CM/SH]	ANS	Eq.I	1.7	6.2		0.18	

(B)

Refolding from	Cysteine ^c	Equation ^b	Amplitudes			Rate constants (min ⁻¹)	
			A ₁	A ₂	A ₃	k ₁	k ₂
D[5-6]	Cys 73	Eq.II	0.70	-0.31	-0.28	0.0034	0.01
D[5-6]	Cys 120	Eq.II	0.64	-0.43	-0.14	0.0086	0.20
D[5-6]	Cys 367	Eq.II	0.23	0.45	0.24	0.013	0.29
D[5-6]	Cys 382	Eq.II	0.30	0.17	0.33	0.11	0.76
D[3CM/5-6]	Cys 11	Eq.I	0.49	-0.41		0.46	
D[3CM/5-6]	Cys 30	Eq.II	0.42	-0.21	-0.14	0.01	0.18
D[3CM/5-6]	Cys 73	Eq.III	0.012	0.00013			
D[3CM/5-6]	Cys 120	Eq.II	0.44	-0.27	-0.10	0.012	0.46
D[3CM/5-6]	Cys 367	Eq.II	0.30	0.44	0.22	0.010	0.21
D[3CM/5-6]	Cys 382	Eq.II	0.31	0.055	0.45	0.018	0.46

DISCUSSION

In former chapters, the author investigated the refolding mechanism of ovalbumin using the urea-denatured protein D[3-4], D[SH] or mixture of disulfide isomers D[mix] as the starting protein samples. It has been demonstrated that most of the urea-denatured ovalbumin molecules can correctly refold via intrachain sulfhydryl/disulfide exchange reactions in an initial burst intermediate I_N (see Scheme 2 in chapter II). The present study has more clearly demonstrated that urea-denatured ovalbumin with a non-native disulfide can correctly refold by use of D[5-6]. As shown Fig. 18A, it is obvious that the disulfide Cys 367-Cys 382 rearranges to native disulfide Cys 73-Cys 120 via intrachain sulfhydryl/disulfide exchange reactions in I_N. Most of the protein refolded from D[5-6] is the same as the native protein in all aspects; spectroscopic analysis (Figs. 13 and 14), trypsin resistance (Fig. 16), melting temperature (Fig. 17) and its disulfide (Fig. 18A). These results lead to the conclusion that ovalbumin, if any, hardly misfolds and correctly refolds without the help of a catalytic reagent or of an enzyme on account of the presence of the flexible refolding intermediate I_N. I_N is flexible enough to rearrange its disulfide with easy.

The intermediate I_N has characterized by use of refolded protein from D[3CM/5-6] in the present study. Intrinsic tryptophan fluorescence and far-UV CD spectra showed that D[3CM/5-6] could refold to an initial burst intermediate I_N (Figs 13 and 14). Analysis by CD ellipticity, trypsin resistance and ANS binding showed that D[3CM/5-6] never recovered its secondary structure and refolded to native state after initial burst phase

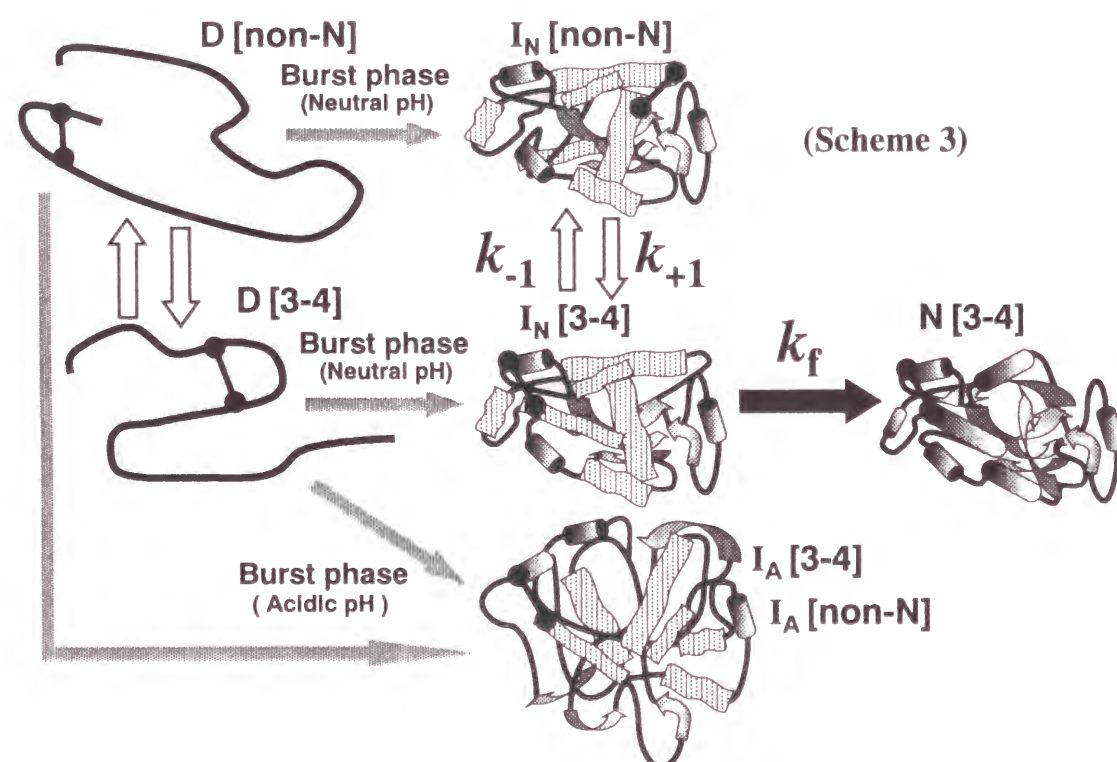
(Figs 16 and 20). We can say that refolded protein from D[3CM/5-6] is the protein stops refolding in I_N state. The intermediate I_N can be characterized that 60 % of secondary structure recovers to native (Fig. 14) and it has no trypsin resistance (Fig. 16), is unstable (Fig. 17) and is flexible enough to occur intrachain sulfhydryl/disulfide exchange reactions (Fig. 18). I_N is, moreover, closed molecule (Fig. 19) and compact near the native state (Table 2). However, increasing of intrinsic tryptophan fluorescence intensity in the D[3CM/5-6] data after initial burst phase is inconsistent with being at a stop in I_N state (Fig. 16). The only inconsistent data is possible to interpret that the different disulfide isomer of I_N may emit the different fluorescence intensity. Increasing the $F_N(t)$ probed by intrinsic tryptophan fluorescence in D[3CM/5-6] (Fig. 16) coincides with increasing Cys 30 and Cys 120 in disulfide-involved cysteine data (Fig. 18B). The first rate constants k_1 obtained by nonlinear least-squares were 0.010; intrinsic tryptophan fluorescence, 0.011; Cys 30, 0.012; Cys 120, respectively. Likewise, disulfide isomers generated from D[3CM/5-6] are exchangeable each other and are equilibrium after 20 h incubation. Therefore, it seems reasonable to suppose that refolded protein from D[3CM/5-6] is regarded as the I_N state protein comprising various disulfide isomers. Another intermediate I_A also has characterized. It recovers 60 % of its secondary structure to native (Fig. 14) and has no trypsin resistance (chapter II) as the same I_N . However, I_A is extend molecule exposed hydrophobic core (Fig. 19 and Table 2) in contrast to I_N .

The present study has demonstrated another important point.

Ovalbumin with non-native disulfide never refolds to native state without rearranging its disulfide to native form in I_N . It is necessary for disulfide-bonded ovalbumin to undergo I_N [3-4] to refold to native state. While disulfide reduced ovalbumin can refold to native protein. These facts lead to the conclusion that the native disulfide Cys 73-Cys 120 is not necessary to refold to native state but a non-native disulfide prevents the intermediate I_N from refolding to native protein. It is probable that ovalbumin cannot refold to native state without correct intra-molecular interaction. The intermediate I_A , which is extended molecule, is impossible to have correct intra-molecular interaction because of unusual charge of side chain at low pH. The intermediate I_N , in spite of its compact conformation near the native state, is also impossible to do because of its non-native disulfide. Consequently, the intermediate I_A and I_N [non-N] never refold to native state. For reasons mentioned above, the author presents Scheme 3 about refolding process of ovalbumin from the urea-denatured state.

Since formation of the secondary structure in protein subjects to the local side chain-side chain interactions, methods for prediction of secondary structure have often used (50-54). On the other hand, it has been evident and widely accepted that formation of the secondary structure subjects to not only the local interactions but also the non-local interactions from the specific tertiary structure, recently (55-58). The specific non-local interactions is greatly regarded at present protein studies; for example, $\alpha \rightarrow \beta$ transition related to prion diseases (59-63). The author considers that in case of ovalbumin, the secondary structure refolding in initial burst phase

may subject to the local interactions because it can recover independent from pH or disulfide types. The remaining secondary structure refolding after initial burst phase may subject to the specific non-local interactions because it cannot recover without correct intra-molecular interaction. The specific non-local interactions may be so rigorously that cannot accept only one mis-paired disulfide in compact molecule near the native.



Schematic refolding of ovalbumin

Urea-denatured ovalbumin D ([3-4]; with native disulfide, [non-N]; with non-native disulfide) refolds to intermediate I_N or I_A whose secondary structure recover about 60% to native protein ($N[3-4]$) through the initial burst phase (gray arrows). I_N , which generates by dilution with neutral pH buffer, is the compact molecule near the native protein whereas I_A , which generates by dilution with acidic pH buffer, is the extended molecule exposed hydrophobic core. I_N is, however, unstable and so flexible as to rearrange its disulfide via sulfhydryl/disulfide exchange reactions (white arrows; k_{+1} and k_{-1} are the rate constants for conversion). $I_N[3-4]$ can refold to native protein with a first-order rate (a black arrow; k_f is the rate constant). $I_N[\text{non-N}]$ never refolds to native protein without undergoing $I_N[3-4]$ via sulfhydryl/disulfide exchange reactions.

SUMMARY

It is generally difficult to investigate the folding mechanism of relatively high molecular proteins with disulfide. In this thesis, refolding process of ovalbumin from urea-denatured state was studied by use of analysis for intrachain disulfide rearrangements and disulfide isomers. Ovalbumin has unique structural characteristics that is four cysteine sulfhydryls along with an intrachain disulfide in a single polypeptide chain. The characteristics enable to analyze the refolding pathways and intermediates with tracing disulfide isomers without using oxidizing agents.

In chapter I, refolding process was investigated using disulfide-bonded and reduced ovalbumin. Two types of refolding intermediates I_N and I_A were detected after initial burst phase that is complete within the mixing dead time of 4ms. I_N , which generates by dilution with neutral pH buffer, refolds to native protein slowly whereas I_A , which generates by dilution with acidic pH buffer, stops refolding. I_A , however, also refolds to native state in case the equilibrium intermediate is placed in a near neutral pH condition. Intrachain sulfhydryl/disulfide exchange reactions occur in I_N . Consequently, non-native disulfide species generate during refolding no matter how refolding is started from only native-disulfide protein. The fact is opposed to the framework model that suggests about mechanism of protein structure construction. The author presents a schematic of refolding pathway of ovalbumin as Scheme 1 by means of kinetic analysis.

In chapter II, refolding process was investigated using mixture of various disulfide isomers of urea-denatured ovalbumin. It was declared that most,

if not all, of the urea-denatured ovalbumin comprises non-native disulfide isomers can correctly refold into the native form via disulfide rearrangements according to Scheme 2.

In chapter III, refolding process was investigated using chemical modified ovalbumin with a non-native disulfide Cys 367- Cys 382. Two refolding intermediates were characterized that both their secondary structure recovered about 60% to native protein but their whole conformations were very different. I_N is the compact molecule near the native protein whereas I_A is the extended molecule exposed hydrophobic core. I_N is, however, unstable and so flexible as to rearrange its disulfide via sulfhydryl/disulfide exchange reactions. Consequently, non-native disulfide species can correctly refold to native protein with disulfide rearrangement to the native pair without the help of a catalytic reagent or of an enzyme. It was, therefore, declared that non-native disulfide species stopped refolding in I_N and never refolded to native protein without disulfide rearrangement to the native pair. The author presented Scheme 3 about refolding process of ovalbumin from urea-denatured state.

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PUBLICATIONS

1. Maki Onda, Eizo Tatsumi, Nobuyuki Takahashi and Masaaki Hirose :

Refolding Process of Ovalbumin from Urea-denature State.

Evidence for the Involvement of Non Productive Side Chain

Interactions in an Early Intermediate.

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2. Maki Onda, Eizo Tatsumi, Nobuyuki Takahashi and Masaaki Hirose :

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3. Maki Onda and Masaaki Hirose :

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